CHARACTERIZATION OF BRAIN TYPE I RECEPTORS FOR ADRENOCORTICOSTEROID HORMONES

By

SEYED MOHAMMAD EMADIAN

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1987

Dedicated to my parents and my wife without whose continuous support this work would not have been possible

ACKNOWLEDGMENTS

The experiments in this dissertation were conducted under the guidance of my mentor and chairman of my supervisory committee, Dr. William G. Luttge. I am indebted to Dr. Luttge for being a superb mentor, teacher, colleague, friend, and for his continuous support throughout the course of my dissertation work. I further would like to extend my gratitude to my other supervisory committee members, Drs. A.J. Dunn, M.J. Fregly, M.B. Heaton and K.T. Shiverick, for their guidance and critical evaluation of my dissertation. I am also grateful to Dr. L.C. Garg for his participation in the discussion of this work and his helpful and valuable contributions during our committee meetings. I would like to thank Dr. C.L. Densmore and Ms. Y.-C. Chou for their helpful comments and evaluation of the results of this work during our laboratory meetings and Ms. Mary E. Rupp and Mrs. Cameron F. Bloom for technical assistance. Last, but not least, I would like to acknowledge the financial assistance provided to me by the Department of Neuroscience and Center for Neurobiological Sciences during the course of my graduate studies.

TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS iii

LIST OF TABLES vi

LIST OF FIGURES vii

ABBREVIATIONS ix

ABSTRACT xii

CHAPTERS

I REVIEW OF THE LITERATURE 1

Control of ALDO Secretion 4
Peripheral Site of Action and Effects of Mineralocorticoids 5
Central Site of Action and Effects of Mineralocorticoids 11
Molecular Mechanisms of Mineralocorticoid Action 18
Mechanisms of Receptor Regulation 26
In Vitro Analysis of Type I Receptors by Radioreceptor Assay 36

II EXPERIMENTAL PROCEDURES 43

Isotopes, Steroids and Other Chemicals 43
Experimental Animals. 44
Buffers 44
Cytosol Preparation 45
Aging 45
Hydrophobic Interaction Chromatography 45
Steroid Binding Determination 46
Cytosolic Protein Concentration 47

III EFFECTS OF POLYHYDRIC AND MONOHYDRIC COMPOUNDS ON THE STABILITY OF TYPE I RECEPTORS FOR ADRENOCORTICOSTEROID HORMONES IN BRAIN CYTOSOL 48

Summary 48
Introduction 49
Materials and Methods 50
Results 50
Discussion 58

Proposed Mechanism of Action of Poly- and Monohydric Compounds 58
Possible Mode of Action of Poly- and Monohydric Compounds on Unoccupied Type I Receptors in Crude Cytosol 64

IV A_3NOVEL EFFECT OF MOLYBDATE ON THE BINDING OF [^{3}H]ALDOSTERONE TO GEL FILTERED TYPE I RECEPTORS IN BRAIN CYTOSOL 70

Summary 70
Introduction 71
Materials and Methods 72
Results 73
Discussion 88

V DIFFERENTIAL INACTIVATION OF TYPE II RECEPTORS FOR ADRENAL STEROIDS IN WHOLE BRAIN CYTOSOL: RECOVERY OF INTACT TYPE I RECEPTORS 93

Summary 93
Introduction 94
Materials and Methods 96
Results and Discussion 97
Effects of DTNB on Unoccupied Type I Receptors 97
Effects of DTNB on Occupied Receptors 101
Effects of DCC Pretreatment of Cytosol on Ligand Binding Capacity of Unoccupied Type I and Type II Receptors 106

VI IN VITRO TRANSFORMATION OF ALDOSTERONE-TYPE I RECEPTOR COMPLEXES TO A DNA-BINDING STATE 122

Summary 122
Introduction 123
Materials and Methods 129
Results 130
Discussion 139

VII CONCLUDING REMARKS 142

REFERENCES 145

BIOGRAPHICAL SKETCH 184

LIST OF TABLES

		Page
Table 2-1:	Buffers	44
Table 4-1:	Binding Parameters for Type I Receptors Obtained from Crude and Gel Filtered Cytosol in the Presence and Absence of 2 mM Molybdate	82
Table 5-1:	Effects of 1 mM DTNB on Whole Brain Cytosolic Unoccupied Type I Receptors	100
Table 5-2:	Effects of Two Consecutive DCC Pretreatments of Cytosol on the Residual DEX Specific Binding	111
Table 6-1:	Comparison of the Binding of ALDO-Type I and DEX-Type II Receptor Complexes to DNA-cellulose Before and After Salt- and/or Temperature-Induced Activation	132
Table 6-2:	Effects of 5 mM Molybdate on the Binding of ALDO-Type I Receptor Complexes to DNA-Cellulose Before and After Salt- and Temperature-Induced Activation	136

LIST OF FIGURES

Page

Figure 3-1: Effects of Polyhydric Compounds on the Stability of Unoccupied Type I Receptors in Whole Brain Cytosol 52

Figure 3-2: Effects of 10% Glycerol on the Stability of Unoccupied Type II Receptors in Whole brain Cytosol 55

Figure 3-3: Effects of Monohydric Compounds on the Stability of Unoccupied Type I Receptors in Whole Brain Cytosol 57

Figure 3-4: Dose-Response Analysis of the Effects of Ethanol on the Binding of ALDO to Type I Receptors in Whole Brain Cytosol 60

Figure 3-5: Scatchard Analysis of the Effects of 10% Ethanol on the Equilibrium Binding Parameters of ALDO Binding to Type I Receptors in Whole Brain Cytosol 62

Figure 3-6: Comparison of the Surface Hydrophobic Properties of ALDO-Type I and DEX-Type II Receptor Complexes on Pentyl-Agarose Columns 68

Figure 4-1: Effects of Gel Filtration, Molybdate and/or Dithiothreitol on the Binding Capacity and Stability of Unoccupied Type I Receptors From Whole Brain Cytosol 75

Figure 4-2: Dose-Response Analysis of the Effects of Gel Filtration and Molybdate on the Binding Capacity and Stability of Unoccupied Type I Receptors from Whole Brain Cytosol 78

Figure 4-3: Effects of Molybdate and/or Gel Filtration on the Equilibrium Binding Parameters of ALDO Binding to Unoccupied Type I Receptors from Whole Brain Cytosol 81

Figure 4-4: Stability of Unoccupied Type I Receptors from Whole Brain Cytosol Following Gel Filtration in the Absence of Molybdate: Effects of Molybdate Addition Following Gel Filtration and Aging 85

Figure 4-5: Effects of Dilution and/or Molybdate on the Binding Capacity of Unoccupied Type I Receptors from Whole Brain Cytosol 87

Figure 5-1: Effects of 1 mM DTNB on Receptors Bound to ALDO in Whole Brain Cytosol 103

Figure 5-2: Effects of DCC Pretreatment and/or Aging of Unlabeled Whole Brain Cytosol on the Binding of ALDO to Type I and DEX to Type II Receptors . . 105

Figure 5-3: Effects of Pretreatment of Whole Brain Cytosol with Various Concentrations of DCC and Different Duration of DCC Pretreatment on the Binding of DEX to Type II Receptors 109

Figure 5-4: Effects of DCC Pretreatment of Whole Brain Cytosol on the Binding of ALDO and DEX to Type I and Type II Receptors in the Presence or Absence of a Steroid Competitor 114

Figure 5-5: Effects of 300 mM KCl in the Presence and Absence of 20 mM Molybdate on the Binding of ALDO to Type I and DEX to Type II Receptors 117

Figure 5-6: Effects of 300 mM KCl on the Specific Binding of ALDO and DEX Prior and Subsequent to DCC Pretreatment 119

Figure 6-1: Comparison of the Surface Hydrophobic Properties of Unactivated and Activated ALDO-Type I Receptor Complexes on Pentyl-Agarose Columns 138

ABBREVIATIONS

AII: angiotensin II

Ach: acetylcholine

ACTH: adrenocorticotropic hormone

ADP: adenosine diphosphate

AIPs: aldosterone-induced proteins

ALDO: aldosterone

ANF: atrial natriuretic factor

ASF: aldosterone-stimulating factor

ATPase: adenosine triphosphatase

B_{MAX}: maximal binding

B_{NS}: nonspecific binding

B_T: total binding

CBG: corticosteriod-binding globulin

CPM: counts per minute

CNS: central nervous system

CORT: corticosterone

CSF: cerebrospinal fluid

DCC: dextran-coated charcoal

DEAE-Cellulsoe: diethylaminoethyl-Cellulose

DEX: dexamethasone

DNA: deoxyribonucleic acid

DNA-C: deoxyribonucleic acid-cellulose

DOC: deoxycorticosterone

DOCA: deoxycorticosterone acelate

DPM: disintegration per minute

DTNB: 5,5'-dithiobis (2-nitrobenzoic acid)

DTT: dithiothreitol

E: erythritol

EEDQ: N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinolone

EG: ethylene glycol

Et: ethanol

G: glycerol

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hn-RNA: heterogeneous nuclear-ribonucleic acid

K_d: equilibrium dissociation constant

MMTS: methyl methanethiosulfonate

MMTV: mouse mammary tumor virus

mRNA: messenger ribonucleic acid

Mt: methanol

NAD^+: nicotinamide-adenine dinucleotide

NADPH: nicotinamide-adenine dinucleotide phosphate

NEM: N-ethylmaleimide

NM: nuclear matrix

P: propanol

PMSF: phenylmethyl sulfonylfluoride

R: ribitol

RPM: revolutions per minute

S: sorbitol

SCO: subcommissural organ

SDS-PAGE: sodium dodocylsulfate-polyacrylamide gel electrophoresis

TA: triamcinolone acetonide

T_m: melting temperature

X: xylitol

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

CHARACTERIZATION OF BRAIN TYPE I RECEPTORS FOR ADRENOCORTICOSTEROID HORMONES

By

Seyed Mohammad Emadian

May 1987

Chairman: William G. Luttge, Ph.D.
Major Department: Neuroscience

Preponderance of evidence suggests that the potent effects of adrenocorticosteroid hormones on a number of physiological and behavioral functions are initiated through stereospecific binding of these steroids with soluble intracellular receptors, followed by the interaction of the steroid-receptor complexes with the genome. Despite the wealth of our knowledge regarding the properties of steroid hormone receptors in general, practical difficulties such as lack of specific available ligands, receptor instability under cell-free conditions, low receptor concentration in whole tissue preparations, etc., have greatly limited our knowledge of properties and regulation of Type I receptors for adrenocorticosteroid hormones.

The work presented in this dissertation has overcome some of these difficulties and describes a number of distinct in vitro properties of these receptors: 1) Unlike Type II (glucocorticoid) receptors, the extraction of unoccupied Type I receptors into an aqueous environment was found to result in a reduction in the binding capacity of these

receptors--possibly by inducing a conformation of the receptors with an infolded hydrophobic steroid-binding pocket. A reduction in hydrophobic interactions by including monohydric compounds in cytosol was shown to result in an increase in the binding capacity of these receptors for the ligand. 2) Although the stabilizing effects of molybdate on steroid receptors is well established, this oxyanion was found to produce a paradoxical effect on the binding capacity of unoccupied Type I receptors in crude and gel filtered cytosol preparations: a reduction in receptor binding capacity in crude, and an increase in receptor binding capacity in gel filtered cytosol was observed in the presence of molybdate. 3) Whereas unoccupied Type II receptors could be inactivated by removal of endogenous sulfhydryl reducing agent from cytosol using dextran-coated charcoal (DCC), Type I receptors appeared insensitive to the effects of dithiothreitol (a potent sulfhydryl reducing reagent) and DCC pretreatment of cytosol (a mild oxidizing condition). The differences between these two receptors were used to inactivate selectively Type II receptors. 4) Lastly, conditions that induced transformation (activation) of glucocorticoid-Type II receptors to a DNA-binding state (i.e., 300 mM KCl and incubation at 22°C) were found to produce minimal activation of Type I receptors. Possible functional and physiological significance of these findings is discussed.

CHAPTER I
REVIEW OF THE LITERATURE

The actions of adrenocorticosteroid hormones on a wide variety of physiological and behavioral functions in mammals are well characterized (for review see Fregly and Luttge, 1982; Luttge, 1983; Rees and Gray, 1983; De Kloet 1984; Munck et al., 1984; Meyer, 1985; Funder, 1986; De Kloet et al., 1986; McEwen et al., 1986b; Munck and Guyre, 1986). As a result of extensive research, significant developments have occurred in the past three decades with regard to the mechanism of action of these hormones (for review see Reich and Scott, 1979; Fanestil and Kipnowski, 1982; Lan et al., 1984; Rossier, et al., 1985; Marver, 1986; Scheidereit et al., 1986; Slater et al., 1986). Although limited experimental evidence suggests that the actions of adrenocorticosteroid hormones may result from direct interactions of these steroids with components of membrane (nongenomic response) (Chaplin et al., 1981; Towle and Sze, 1983), the bulk of evidence suggests that steroid hormones, in general, exert their effect via a stereospecific binding with soluble intracellular protein receptors. The resulting steroid-receptor complexes undergo certain physicochemical changes that increase the overall affinity of the complexes for nuclei, chromatin and DNA and other polyanions (for detail see Chapter VI). This "transformation" process (also known as "activation") allows for a direct interaction of the transformed complexes with nuclear acceptor site(s) which in turn modulate the transcription of certain mRNAs and hence cellular protein synthesis. It is important to note that steroid

hormone receptors are the only regulators of gene expression in the eukaryotic cells thus far described. Indeed, recent studies have characterized specific palindromic sequences within the DNA molecule--hormone response elements--to which the transformed steroid receptors bind in order to modulate the transcription of certain mRNAs (e.g., Arnemann et al., 1987; Chambone et al., 1987; Drouin et al., 1987; Karin et al., 1987; Miesfeld et al., 1987).

Long before isolation and purification of aldosterone (ALDO) (Grundy et al., 1952; Simpson and Tait, 1952), the most potent naturally occurring mineralocorticoid hormone, it was known that blood borne "factors" controlled electrolyte balance within the body (Addison, 1855). Lucas (1926) provided the first evidence for the adrenal cortical regulation of mineral metabolism by adrenalectomizing dogs. However, despite these and many other preliminary observations, the discovery of ALDO production by zona glomerulosa of the adrenal glands came long after the discovery of the main corticosteroid hormones produced by the zonae fasciculata and reticularis. The earlier investigators attributed hyponatremia, hypochloremia, acidosis, hyperkalemia, and other symptoms associated with Addison's disease to the lack of deoxycorticosterone (DOC) secretion (Thorn et al., 1942). Despite the potent mineralocorticoid activity of DOC, this theory was discredited when extracts from adrenal glands were found to contain only minute amounts of DOC. The discovery of ALDO came about some years later as a result of improved quantitative bioassay and chromatographic techniques (Grundy et al., 1952; Simpson and Tait, 1952; Simpson et al., 1954; Simpson and Tait, 1955).

Because of the potent action of ALDO on mineral metabolism (for detail see below), the high affinity binders for this steroid extracted from the kidney were dubbed as "mineralocorticoid receptors." However, since the endogenous glucocorticoid, corticosterone (CORT), was found to have comparable affinty for these receptors (see below), a less ambiguous term, Type I receptors, will be used in this dissertation to refer to these receptors. This terminology will also be used to distinguish these receptors from the lower affinity, higher capacity ALDO binders, the Type II (glucocorticoid) receptors. Although the principal target tissues for the action of ALDO in mammals have been considered to be the kidney, intestines and salivary glands, because electrolyte exchange occurs throughout the organism, other tissues may also be considered as targets for ALDO. This claim is further supported by the fact that Type I receptors have been identified in the pituitary gland (Moguilewsky and Raynaud, 1980; De Nicola et al., 1981), parotid gland (Funder et al., 1972), mammary gland (Quirk et al., 1983), lung (Krozowski and Funder, 1981), bovine ciliary body (Starka et al., 1977), rabbit lens epithelium (Hampl et al., 1981), human ocular lens (Hampl, et al., 1984; Starka et al., 1986), whole brain cytosol (Anderson and Fanestil, 1976; Emadian et al., 1986), various brain regions (Moguilewsky and Raynaud, 1980; De Nicola et al., 1981; Veldhuis et al., 1982b; Coirini et al., 1983), spinal cord (Orti et al., 1986), human mononuclear leukocytes (Armanini et al., 1986), rat epididymis (Hinton and Keefer, 1985; Pearce et al., 1986a), "arterial walls" (Kornel, 1981; Kornel et al., 1982a & b) and rat cochlear and vestibular organs (Rarey and Luttge, 1987).

Control of ALDO Secretion

In mammals, several known physiological modulators of ALDO secretion from the adrenal cortex include the renin-angiotensin II system, potassium ion and adrenocorticotropic hormone (ACTH). An extensive body of research has implicated the renin-angiotensin II system as the principal regulator of ALDO secretion (e.g., Kaplan, 1965; Davis, 1975; Fraser et al., 1979). Although ACTH stimulation of ALDO steroidogenesis via the adenylate cyclase cascade mechanism is well characterized, ACTH is apparently not involved in the maintenance of ALDO secretion under basal conditions. Note, for example, that suppression of ACTH secretion with dexamethasone (DEX), a synthetic glucocorticoid, and other manipulations (e.g., hypophysectomy) does not alter the circadian rhythm of ALDO secretion (Fraser et al., 1979). Furthermore, infusion of ACTH leads to sustained increases in plasma ALDO concentration, suggesting that ACTH may mediate only acute increases in ALDO secretion physiologically (Kem et al., 1975).

A number of other factors including aldosterone-stimulating factor (ASF, secreted by the anterior pituitary) (Carey and Sen, 1986); atrial natriuretic factor (ANF, a family of peptides isolated from the atria of rat, human and other species) (Chartier et al., 1984; Takagi et al., 1986; Aguilera, 1987; Naruse et al., 1987; Sagnella et al., 1987); acetylcholine, ACh (Kojima et al., 1986); dopamine (Carey and Drake, 1986; for review see Campbell et al., 1981); vasopressin (Woodcock et al., 1986) and serotonin (Matsuoka et al., 1985) have been recently implicated in the regulation of ALDO secretion. Large reductions in the concentration of sodium in serum have also been shown to stimulate ALDO

secretion; however, the physiological role of this hyponatremia-induced ALDO secretion awaits further exploration (Davis, 1975).

Peripheral Site of Action and Effects of Mineralocorticoids

As mentioned above, the main peripheral action of ALDO is to maintain electrolyte balance in the organism. A secondary action is to help maintain proper blood volume and pressure. These actions of ALDO are achieved primarily through reabsorption of sodium and hence water (due to osmotic activity of sodium) and excretion of potassium and hydrogen ions by the kidney. In other words, an increase in the circulating levels of ALDO results in a decrease in the ratio of sodium to potassium in urine, hence an increase in plasma and extracellular fluid volume. An ALDO-mediated reduction in the Na/K ratio is also reported in saliva, sweat and feces.

The antinatriuretic and kaliuretic effects of ALDO on kidney are well established. Due to a remarkable anatomical complexity and specialization in this organ (for review see Jacobson, 1981), the exact sites at which these effects are mediated remained controversial for some time (Knox et al., 1980). Because ALDO, deoxycorticosterone acetate (DOCA, an acetate derivative of DOC) and other steroids with mineralocorticoid activity were found to influence electrolyte balance without changing renal plasma flow and glomerular filtration rate (Barger et al., 1958; Ganong and Mulrow, 1958), the distal tubule was postulated to be the main locus of mineralocorticoid action (Hierholzer et al., 1965; Wright and Giebisch, 1978). Studies with isolated, perfused tubules from rabbit kidneys, however, provided evidence suggesting that mineralocorticoids (administered acutely or chronically) increase sodium reabsorption and potassium secretion in the cortical

collecting tubules and not the distal tubules (O'Neil and Helman, 1977; Schwartz and Burg, 1978; Stokes et al., 1981). Furthermore, mineralocorticoids were also found to increase discretely the transtubular electrical potential (lumen negative) across the cortical collecting tubules (Gross et al., 1975; Gross and Kokko, 1977), a finding not confirmed in the study by Schwartz and Burg (1978). Determination of pH profile along the rat papillary collecting duct by micropuncture technique suggested the action of ALDO on distal papillary collecting duct (Higashihara et al., 1984). In addition to the possibility that ALDO may influence multiple segments of the nephron, the uncertainty as to the site of action of mineralocorticoids in kidney and other target tissues may also be due to species, tissue and/or methodological differences between the aforementioned studies. For example, in contrast to the results obtained from the rabbit nephron, ALDO was found to normalize sodium transport in the rat distal tubule (Hierholzer and Wiederholt, 1976; Horisberger and Diezi, 1983) without changing the transtubular potential (Hierholzer and Wiederholt, 1976). Moreover, ALDO action at receptor sites other that Type I receptors cannot be ruled out. Note that the administration of DEX (a specific synthetic glucocorticoid) to adrenalectomized rats has been shown recently to increase Na^+/H^+ exchange activity in isolated renal brush border membrane vesicles (Kinsella et al., 1985). Other investigators, however, reported that glucocorticoids have kaliuretic and not antinatriuretic effects (e.g., Bia et al., 1982; Campen et al., 1983; for review see Field and Giebisch; 1985). The use of <u>in situ</u> microperfusion of single superficial distal tubles in adrenalectomized rats revealed no direct effect of DEX on potassium secretion by the

microperfused tubule; however, an overall increase in urinary flow and sodium excretion was a common finding in these studies (Field et al., 1984). In contrast, infusion of ALDO under identical conditions led to a 90% stimulation of potassium secretion by the microperfused tubule and a marked reduction in the kidney sodium excretion and urinary flow rate. No apparent effect on kidney potassium excretion was observed under this latter condition (Field et al., 1984). It is postulated that mineral exchange represents the net result of direct and synergistic actions of the steroids on renal tubules plus secondary effects mediated by changes in urinary flow rate (Field et al., 1984). Parenthetically, recent studies on the effects of corticosteroids on Na^+ transport in distal colon suggest a different cellular mechanism for ALDO and DEX (Na^+ transport in response to ALDO, but not DEX, was found to be amiloride sensitive) (Jorkasky et al., 1985). Furthermore, since basal plasma levels of ALDO (5 ng/dl) in the presence of potassium loading significantly increased transepithelial potential difference and the area of the basolateral membrane in the late distal convolution (a characteristic feature of "potassium adaptation" when pharmacological doses of ALDO are used in potassium-loaded adrenalectomized animals), Hirsch et al. (1984) postulated a differential mechanism of ALDO action on different segments of nephron. Note that neither the acute nor chronic administration of ALDO in the absence of potassium loading has any effect on the late distal convolution, whereas this latter treatment increased surface density of the basolateral membrane in initial collecting tubule cells (Hirsch et al., 1984).

Autoradiographic and binding assay studies focusing on the regional distribution of Type I receptors in various segments of renal nephron have yielded fruitful results regarding the potential site(s) of mineralocorticoid action along the nephron. Radioreceptor assays of cytosolic fractions obtained from rat kidney cortical tubules enriched in proximal or distal segments revealed a higher concentration of [^{3}H]ALDO binding in the latter (in the presence or absence of a glucocorticoid competitor), although the precise location of Type I receptors along the nephron was not identified (Scholer et al., 1979). In the rabbit nephron, high specific [^{3}H]ALDO binding ($>10^{-16}$ mol/cm tubule length) was found in the branched, cortical and outer medullary collecting tubules, whereas specific binding was negligible in the proximal tubules, pars recta, medullary thick ascending limb, cortical thick ascending limb and distal convoluted tubules ($<10^{-17}$ mol/cm tubule length) (Doucet and Katz, 1981a, Farman et al., 1982a). In contrast, using CORT as a ligand, receptor binding was demonstrated throughout the rat nephron, albeit the greatest concentration was found in the cortical collecting tubules (Lee et al., 1983). Autoradiographic studies are consistent with radioreceptor assays, in that, specific ALDO binding was observed along the distal and cortical collecting tubules (Vandewalle et al., 1981; Farman et al., 1982b; Farman and Bonvalet, 1983), but not in the glomeruli (Farman et al., 1982c).

Recent research on the endocrine basis of experimental hypertension suggest a role for a renally-independent (Berecek and Bohr, 1978; Onoyama et al., 1979) intraarterial mechanism of mineralocorticoid action (Kornel, 1981; Kornel et al., 1982a & b; for review see Kotchen and Guthrie, 1980). Chronic administration of DOCA was found to

increase the turnover of arterial tissue potassium and chloride ions and to increase cell membrane "leakiness" to Na^+ (Friedman et al., 1975; Jones and Hart, 1975; Friedman and Friedman, 1976). In agreement with these results, ALDO was found to increase significantly sodium flux along the porcine arterial wall (a tissue with morphological resemblance to human arterial wall) (Llaurado et al., 1983). An increase in peripheral resistance and vascular reactivity was also reported in the DOCA-treated animals (Hansen and Bohr, 1975; Berecek and Bohr, 1977 & 1978). Treatment of normal subjects with the synthetic mineralocorticoid, 9-α-fluorocortisol, for several weeks resulted in a rise in blood pressure in those subjects, an effect which could be reversed by administration of spirolactone (a mineralocorticoid antagonist) (Nicholls et al., 1979).

Clinically, steroid-induced hypertension is usually associated with three types of adrenal disorders. 1) Excess secretion of aldosterone from an autonomous adrenocortical adenoma in primary hyperaldosteronism (first described by Conn, 1955). This hypertension can be remedied usually through surgical procedures and/or by administration of antimineralocorticoids (e.g., spironolactones) (Lim et al., 1986). 2) Cushing's syndrome, characterized by an excessive secretion of cortisol (a glucocorticoid) and DOC, is accompanied by hypertension in about 80-90% of the patients (Gomez-Sanchez, 1982). 3) In patients with 11-β-dehydrogenase and 5α-reductase ("apparent mineralocorticoid excess," a juvenile disease characterized by low or undetectable levels of renin and low plasma ALDO, accompanied by hypokalemia, Monder et al., 1986) and 17α-hydroxylase deficiency (where there is an increase in ACTH-induced ALDO production) there is a high incidence of hypertension.

The exact mechanism by which mineralocorticoids induce hypertension awaits further elucidation. However, since mineralocorticoids can influence electrolyte distribution within the arterial walls by increasing sodium transport and since "specific" mineralocorticoid receptors along the arterial, but not the venous, walls are known to exist (Kornel, 1981; Kornel et al., 1982a & b; Moura and Worcel, 1984), there is strong support for an intraarterial, mineralocorticoid-induced, receptor-mediated basis for this form of experimental hypertension. Studies investigating the effects of ALDO, CORT and DOCA on hypertension in rats suggests that a common mediator of steroid-induced hypertension may be an increase in extracellular fluid and plasma volume (Haack et al., 1977; Ishii et al., 1985). Although aldosterone has been implicated in the pathogenesis of essential hypertension in experimental animal models (e.g., Nowaczynski et al., 1983), the role of mineralocorticoids in essential hypertension is still a matter of controversy (for review see Fraser et al., 1981; Gomez-Sanchez, 1982). Recently, a central nervous system mediated mechanism of ALDO induced-hypertension has been suggested (Gomez-Sanchez, 1986) (for detail see below).

Some other peripheral effects of mineralocorticoid hormones include a redistribution of potasium within the body in addition to the kaliuretic effect discussed above (Young, 1979; Young and Jackson, 1982); a reduction in the concentration of zinc in plasma (Latman et al., 1984); an increase in renal cortical and medullary citrate synthase activity (Kinne and Kirsten, 1968) and synthesis (Law and Edelman, 1978b), a phenomenon independent of sodium transport (at least in cultured cells) (Johnson and Green, 1981); an induction of renal

flavokinase enzymic activity (Trachewsky et al., 1985); an increase in thymidine kinase synthesis in immature rat kidney (Bukhari et al., 1985); an increase in guanylate cyclase activity in liver, kidney, heart, lung, spleen and ilium (Vesely, 1980); an increase in isocitrate dehydrogenase and glutamate-oxaloacetate transaminase activity in rat kidney and toad urinary bladder (Kinne and Kirsten, 1968; Kirsten et al., 1968; Kirsten et al., 1970; Kirsten and Kirsten, 1972); an activation of intestinal mucosal carbonic anhydrase in adrenalectomized rats (Scott and Sapirstein, 1975; Sapirstein and Scott, 1975; Voute and Meier, 1978; Suzuki, 1981; Suzuki et al., 1983; Suzuki and Ozaki, 1984); an increase in Na-K-ATPase activity along the nephron (Katz et al., 1979; Garg et al., 1981; Le Hir et al., 1982; O'Neil and Dubinsky, 1984; for detail see below); an increase in intestinal Mg^{2+}-HCO_3^--ATPase activity (Suzuki, 1981; Suzuki et al., 1983); a diminution of bile flow and liver uptake and a decrease in biliary excretion of sodium and water (Afifi et al., 1979; Afifi, 1981); an increase in red blood cell sodium uptake (Rettori et al., 1969; Afifi et al., 1979; Stern et al., 1983) and a non-genomically-induced increase in membrane phospholipid polyunsaturated fatty acid content (Goodman, 1981).

Central Site of Action and Effects of Mineralocorticoids

Although specific macromolecular binding to ALDO in whole brain cytosol was first described by Anderson and Fanestil (1976), prior to and since this discovery investigators have implicated the involvement of mineralocorticoids in the central nervous system (CNS) electrolyte balance (Woodbury, 1958); neuronal electrical activities (Kraulis et al., 1975); feedback mechanisms involving ALDO secretion (Dundore et al., 1984; Birmingham et al., 1974; Grizzle and Dunlap, 1984);

inhibition of CORT-induced serotonin turnover in dorsal hippocampal and raphe areas (by inhibiting [^{3}H]CORT nuclear uptake) (De Kloet et al., 1983); inhibition of brain growth (Devenport, 1979); influences on glial-cell function (Beaumont, 1985) and inhibition of CORT-induced exploratory behavior (Veldhuis et al., 1982a). In addition, mineralocorticoids have been shown to augment passive avoidance responses in adrenalectomized and adrenally intact "pre-stressed" rats, an effect not observed with CORT administration (Weiss and Gray, 1973).

Although mineralocorticoid-induced hypertension by actions at renal levels is well documented (see above), early reports implicated a role for mineralocorticoid action on brain regulation of experimental hypertension in rats (e.g., Finch et al., 1972; Zamir et al., 1979). Recently, it was shown that minute doses of ALDO administered intracerebroventricularly produce a significant rise in blood pressure (Gomez-Sanchez, 1986). Infusion of ALDO in the presence of an equimolar concentration of prorenone (a spironolactone with antimineralocorticoid activity) antagonized the pressor response to ALDO. Studies of this nature provide strong circumstantial evidence for hypertensinogenic effects of mineralocorticoids directly within the CNS; however, the exact site(s) of action of mineralocorticoids on the CNS regulation of hypertension remains to be elucidated.

Studies investigating the endocrine basis of salt appetite implicate a central role for mineralocorticoid action (Fregly and Waters, 1966; Wolf and Handal, 1966; Weisinger and Woods, 1971; McEwen et al., 1986b). The classical work by Richter (1936) showed that adrenalectomy was accompanied by sodium appetite in rats and that treatment of adrenalectomized rats with DOCA reduced sodium intake to

control levels (Richter, 1956). Similarly, a recent report by McEwen et al. (1986b) showed that the administration of ALDO supressed salt intake in adrenalectomized rats as measured in a two-bottle preference test paradigm (findings that replicate the earlier report by Fregly and Waters, 1966). The supression of salt appetite in response to ALDO could be inhibited by co-administration of a molar excess of RU 28318 (a specific synthetic antimineralocorticoid). It is noteworthy that this effect of ALDO could not be mimicked by CORT, a proposed physiological ligand for brain Type I receptors (for review see Funder, 1986) (for more detail see below). In fact, only concentrations of CORT 500-fold in excess of ALDO were able to inhibit the action of ALDO (McEwen et al. 1986b).

In sheep deprived of sodium for 22 h, infusion of 500 mM Na^+ via a lateral ventricular approach led to a marked reduction in salt intake compared to animals that received 150 mM Na^+ (Weisinger et al., 1979). It is postulated that the modulation of Na^+ concentration in cerebrospinal fluid (CSF) may translate into changes in the concentration of Na^+ in hypothalamic neurons subserving salt appetite (Weisinger et al., 1979). Similarly, an earlier investigation by Richter (1956) showed that low concentrations of sodium in plasma stimulate salt appetite. The author (Ritcher, 1956) implicated (but did not show) possible involment of hypothalamic neurons. Other CNS structures have also been implicated in the regulation of salt intake; for example, electrolytic lesions centered in the thalamic gustatory subnucleus of the ventral posterior complex were shown to impair sodium intake in adrenalectomized rats (Wolf and Dicara, 1974). It is noteworthy that the impairment in salt appetite following such lesions

may be unrelated to the gustatory system since neocortical ablations which resulted in extensive retrograde cell degeneration in this diencephalic structure had no effect on salt appetite (Wolf et al., 1970). Becuase other en passage fibers are damaged by the thalamic lesion (e.g., ascending reticular pathways to the intralaminar nuclei, ascending noradrenergic pathways to the hypothalamus and forebrain, etc.), the involvement of other structures could not be ruled out. Although a specific center(s) in the CNS through which mineralocorticoids may mediate salt appetite and/or other functional responses is (are) yet to be described, it is suggested that the actual mechanism of salt appetite induction may be complex and involve a synergistic association of a number of factors (Fregly and Rowland, 1985).

Since studies of the regional distribution of specific ALDO receptors within the CNS have revealed distinct localization in certain limbic structures such as the hippocampus, hypothalamus, indusium griseum and amygdala, these brain regions have been implicated as possible candidates for the central site of action of ALDO (Ermisch and Ruhle, 1978; Moguilewsky and Raynaud, 1980; De Nicola et al., 1981; Veldhuis et al., 1982b; Coirini et al., 1983; Birmingham et al., 1984; Reul and De Kloet, 1985 & 1986). However, co-administration of "stress levels" of CORT with ALDO was found to suppress preferentially ALDO uptake by hippocampal formation, amygdala and septum, having little effect on ALDO suppression of salt appetite or ALDO uptake in circumventricular organs (McEwen et al., 1986b). Furthermore, bilateral hippocampectomy, which depleted specific [^{3}H]ALDO binding in the residual structure by 80%, had no effect on the development of salt

appetite following adrenalectomy in these hippocampectomized rats (Kim, 1960; Magarinos et al., 1986). The increase in salt intake in adrenalectomized-hippocampectomized animals was found to be suppressible by administration of ALDO (Magarinos et al., 1986). These studies thus suggest, at least in rats, that the hippocampus may not be the target organ for the action of ALDO on salt appetite as elicited by adrenalectomy.

McEwen et al. (1986b) have concluded that one or more of the circumventricular organs, the dentate gyrus, Ammon's horn and/or subiculum may be the most plausible candidates for the action of ALDO on salt appetite since these structures show significant [^{3}H]ALDO uptake even in the presence of high circulating levels of CORT. This notion is consistent with the theory that angiotensin II and ALDO act synergistically to regulate salt intake (Epstein, 1982; Fluharty and Epstein, 1983; Stricker, 1983; Sakai et al., 1986), and that some of these circumventricular organs appear to contain angiotensin II receptors that can be modulated by exposure to ALDO (Wilson et al., 1986). Recently, it has been proposed that ALDO and angiotensin II (AII) may mediate thirst and salt appetite by influencing the activities of septal neurons (Stricker, 1984). Lesions of the septal area in hypovolemic rats were found to produce sensitization to the sodium appetite-eliciting effects of AII; however, this was only seen in the presence of ALDO (Stricker, 1984). Although these studies reflect a synergism between the two hormonal systems in inducing electrolyte and water balance, it must be kept in mind that, in reality, water and electrolyte homeostasis may involve complicated interactions of a number of other systems (for review see Fregly and Rowland, 1985).

Furthermore, limited experimental evidence comparing adrenalectomized mice, hamsters and rats suggests clear differences in the control of salt appetite in these different species.

A developing area of research regarding mineralocorticoid action on the CNS is concerned with the role of a poorly understood CNS structure, the subcommisural organ (SCO). The intraperitoneal or subcutaneous administration of extracts from SCO was found to induce antinatriuresis in dogs (Gilbert, 1963). Palkovits and Foldvari (1963) reported an increased nuclear volume in the cells of the adrenal glomerulosa indicating increased cellular activity following intraperitoneal injection of SCO extracts. In contrast, lesions of the SCO decreased nuclear volume as well as the width of the zona glomerulosa in rats (Palkovits et al., 1965). An in vitro examination of the zona glomerulosa cells from rats with SCO lesion revealed a marked reduction in the rate of ALDO secretion (Palkovits et al., 1965). Furthermore, mineralocorticoids reduced, and adrenalectomy or chronic dehydration increased cellular activity in the SCO (Palkovits, 1968). Together, these observations have led investigators to associate SCO with salt and water homeostasis (for review see Ziegels, 1976).

Recently, Dundore et al. (1984) found that when ALDO was administered in the "general vicinity" of the SCO (note that the injections were performed via a cannula placed in the pineal recess above the rostral two-thirds of the SCO), it produced a natriuresis (an action opposite to those of blood-borne ALDO discussed above) and a "site-specific" (related to cannula placement) reduction in the cross-sectional area of the adrenal medulla without affecting cell density. It is proposed, but not conclusively shown, that ALDO acts

upon SCO cells (and possibly other structures and cells in the vicinity of SCO due to diffusion) to inhibit the trophic effects of SCO on adrenal glomerulosa cells. This, in turn, would result in an increased urinary Na/K ratio (Dundore et al., 1984). Although an attractive hypothesis, this study fails to show a change in plasma concentration of adrenocorticosteroids following intracerebral administration of ALDO. Furthermore, intracerebral administration of ALDO was found to have no significant effect on the cortical (steroid producing) cells of the adrenal gland when examained histologically (Dundore et al., 1984). In light of the above discrepancies and the fact that Crow (1967), who made discrete lesions encompassing all or parts of the SCO observed no changes in urine volume and concentration, it is apparent that the exact role of the SCO in electrolyte metabolism awaits further experiments. It is conceivable that research investigating 1) the direct effects of SCO lesions on plasma levels of corticosteroids, 2) the localization within the SCO of specific mineralocorticoid receptors by autoradiographic and radioreceptor assay techniques and 3) the isolation and purification of SCO "factor(s)" responsible for the aforementioned effects is necessary to establish the physiological role of the SCO, and its secretory factors in salt and water homeostasis.

Some other centrally mediated actions of mineralocorticoid hormones include the regulation of food intake and maintenace of body weight, (Devenport and Devenport, 1983; Devenport et al., 1983; Kenyon et al., 1984); modulation of hypothalamic, thalamic and septal angiotensin II receptor binding capacity (Wilson et al., 1986) and increase in cerebral guanylate cyclase activity (Vesely, 1980).

Molecular Mechanisms of Mineralocorticoid Action

Recent *in vitro* radioreceptor assay and ligand competition techniques suggest that in cytosol lacking extravascular CBG, Type I receptors have an equal affinity for ALDO and CORT (for review see Funder, 1986). It is further suggested that some actions of CORT on the CNS may be mediated through Type I receptors (e.g., De Kloet et al., 1986; Magarinos et al., 1986). However, because in this dissertation the emphasis is placed on the role of Type I receptors in electrolyte homeostasis, this section will focus primarily on our current understanding of the molecular mechanisms of action of ALDO and other physiological mineralocorticoids.

Research on the mechanism of mineralocorticoid action has drawn tremendous benefit from studies carried out using isolated nephrons, the A6 cell line derived from the *Xenopus* kidney epithelial cells and the sodium transporting amphibian epithelia (toad urinary bladder and frog skin). These biological preparations can be maintained viable for several hours at room temperature in incubation media of simple composition. The simple morphological properties of these models render them ideal in the study of the mechanism(s) of mineralocorticoid action; however, it is noteworthy that although aldosterone is the naturally occuring mineralocorticoid produced by the interrenal gland in anurans (Carstensen et al., 1961; Crabbe, 1961), the "mineralocorticoid response," at least in toad bladder appears to be mediated through interactions with Type I as well as other glucocorticoid specific receptors (Geering et al., 1985). Even though the authors dubbed these other receptors as "type 2" glucocorticoid receptors, the possibility

they may be Type IB (for more detail see below) and thus distinct from classical glucocorticoid (Type II) receptors should also be considered.

In the early 1960's, Williamson (1963) and Edelman and co-workers (1963) independently proposed the classic two-step genomic mode of ALDO action. The first step was the binding to specific soluble intracellular receptors, following which, as a second step, the steroid-receptor complexes would translocate into the nucleus where they would interact with the cellular genomic machinery (for a discussion of the nature of the nuclear acceptor site see Chapter VI). It is this latter step that was proposed to result in the modulation of synthesis of a number of structural and regulatory proteins. Several lines of evidence appeared later in favor of this proposal. For instance, Sharp and colleagues (1966b) were the first to report saturable ALDO binding sites (receptors) in the toad bladder. Marver et al. (1974) later reported that the mineralocorticoid antagonist, spirolactone (SC-26304), when complexed with these receptors, had little or no affinity for nuclear acceptor sites in vivo and in vitro. More recently, the response to ALDO in the rat kidney was shown to depend clearly on the ontogeny of Type I receptors in this target organ (Stephenson et al., 1984a). Finally, it was possible to correlate the magnitude of epithelial sodium transport with the binding of ALDO-receptor complexes to chromatin and the increases in the synthesis of a specific class of nonmethylated mRNAs and of proteins (Rossier et al., 1974). In this regard, actinomycin-D (a DNA intercalator which binds specifically to G--C base pairs and protrudes into the major grooves) was shown to block ALDO-stimulated sodium transport by inhibiting new mRNA and protein synthesis (Lahav et al., 1973; Rossier et al., 1974; Horisberger and

Diezi, 1984). Subsequent to these observations a number of studies replicated the results described (for review see Edelman and Marver, 1980; Fanestil and Park, 1981; Rossier et al., 1985; Garty, 1986; Marver, 1986) thus further confirming the original model proposed by Edelman et al. (1963) and Williamson (1963).

It is now well established that mineralocorticoids, in general, are capable of inducing the synthesis of a group of proteins in target tissues generally referred to as aldosterone-induced proteins (AIPs). In the rat kidney and toad urinary bladder, proteins with a variety of molecular weights have been reported to exhibit increased incorporation of radiolabeled precursor amino acids as a result of exposure to aldosterone (Law and Edelman, 1978a & b; Geheb et al., 1981; Reich et al., 1981; Yang et al; 1981; Blazer-Yost et al., 1982; Geering, et al. 1982; Geheb et al., 1983; Geheb et al., 1984). However, the mechanism by which AIPs participate in the mineralocorticoid-induced physiological and functional consequences such as sodium transport still remains a matter of debate. In fact, only recently was it shown that some AIPs can be immunoprecipitated by polyclonal antibodies specific for the α- and β-subunits of Na-K-ATPase (Geering et al., 1982).

Current thinking on the role of AIPs, as related to the action of mineralocorticoids, points to three distinct, but not necessarily independent, theories: 1) the sodium pump theory, which argues for increased sodium pump activity as a result of either activation of pre-existing pumps or an increase in the synthesis of new pumps; 2) the sodium permease theory, which holds that AIPs are involved in the facilitation of passive sodium entry across the plasma membrane and 3) the energy theory, which associates AIPs with the augmentation of

cellular energy, presumably by enhanced mitochondrial oxidative phosphorylation. Although each of these theories has some experimental support, thus far none has gained universal acceptance. It is important to emphasize that the involvement of AIPs may not be limited to only one of these mechanisms exclusively; rather the effects of mineralocorticoids may reflect the contribution of AIPs to some combination of these three general mechanisms. In this regard, Reich and Scott (1979) proposed a unifying hypothesis that includes all three of the above theories (for recent reviews see Rossier et al., 1985; Garty, 1986; Marver, 1986).

Considerable controversy still surrounds the pump theory. In the past decade, a large number of laboratories has investigated the mineralocorticoid induction of Na-K-ATPase activity (e.g., Aperia et al., 1981; Doucet and Katz, 1981b; Garg et al., 1981; Petty et al., 1981; Geering et al., 1982; Osore and Gilbert, 1982; Cortas et al., 1983; El Mernissi and Doucet, 1983; Stern et al., 1983; Aperia and Larsson, 1984; El Mernissi and Doucet, 1984; O'Neil and Dubinsky, 1984; Park and Edelman, 1984a & b; Geering et al., 1985; O'Neil and Hayhurst, 1985; Girardet et al., 1986; Johnson et al., 1986; Palmer and Speez, 1986). As a result of this intensive investigation, it is now well established that following adrenalectomy there is a significant reduction in Na-K-ATPase activity in nephrons; however, some investigators find complete restoration of the enzymatic activity following administration of ALDO (Petty et al., 1981, El Mernissi and Doucet, 1983; El Mernissi and Doucet, 1984), whereas others find little or no effect of ALDO on the restoration of enzyme activities in the target tissues investigated (Doucet and Katz, 1981b).

In addition to this discrepancy in results, the studies cited above also fail to prove that sodium pump activity plays a significant role in the action of mineralocorticoids on transepithelial or nephron sodium transport. Indeed, it is suggested that the observed increase in Na-K-ATPase activity, at least in some target tissues, may be due to interaction of mineralocorticoids with Type II receptors, since long-term pharmacological doses of steroids are often required to restore Na-K-ATPase activity to control levels. Recently, a number of investigators (Rayson and Lowther, 1984; El Mernissi and Doucet, 1984; Garg et al., 1985) reported that they were able to restore the adrenalectomy-induced reduction in Na-K-ATPase activity in various segments of rabbit nephron through DEX administration. Even though similar results were reported by earlier investigators (Fisher et al., 1975), the small doses of DEX used in the former reports (Rayson and Lowther, 1984; El Menissi and Doucet, 1984; Garg et al., 1985), provide stronger evidence that the stimulation of the Na-K-ATPase was probably through Type II receptors. Note that although the radioreceptor assay technique suggests that DEX binding to Type I receptors may be negligible, the pharmacological doses of DEX administered in some of these studies may allow sufficient interaction of this glucocorticoid with these receptors to mediate mineralocorticoid action.

In contrast to this suggestion, El Mernissi and Doucet (1984) have shown that ALDO administration increases the synthesis of new Na-K-ATPase in the collecting tubules, whereas DEX stimulates the activity of the pre-exisiting pumps in the thick ascending limb and distal convoluted tubules. Thus, these results suggest that ALDO and DEX may act through differential mechanisms at different portions of the

nephron. A differential action of ALDO in the rat proximal and distal colon has also been described: ALDO response in distal, but not proximal, colon could be inhibited by amiloride (an acylguanidine diuretic that inhibits influx of Na^+ through the apical membrane) (Hirsch et al., 1985). In contrast, in vitro studies evaluating short-circuit current in colon from adrenalectomized rats suggest that although ALDO may be the physiological regulator of Na^+ transport, glucocorticoids may also mediate amiloride-sensitive sodium transport (Will et al., 1985). Geering et al. (1985) maintain that, at least in toad urinary bladder, ALDO binding to both Type I and Type II receptors is required for full mineralocorticoid response.

It is also conceivable that DEX actions on Na-K-ATPase activity may be mediated through a population of receptors distinct from Type I and Type II receptors called Type IB. The presence of these receptors in a number of tissues including the rat liver (Litwack and Rosenfield, 1975), colon (Bastl et al., 1984) and kidney (Litwack and Rosenfield, 1975; Markovic et al., 1980; Mayer et al., 1983) has been confirmed (for review see Mayer and Litwack, 1983). These receptors have also been isolated from mouse lung cytosol (Goldman and Katsumata, 1986) and were implicated to mediate the known anti-inflamatory and teratogenic actions of DEX and phenytoin (Katsumata et al., 1985). Some investigators, however, have questioned the polymorphic nature of these receptors and have argued that these atypical receptors may represent a proteolytic fragment(s) generated from Type II receptors under in vitro conditions (e.g., Sherman et al., 1983; Reichman et al., 1984). More recently, Eisen et al., (1986) showed that kidney cytosol prepared in the absence, but not the presence, of molybdate (for detail of possible

mechanism of action of this oxyanion see Chapter IV) showed specific glucocorticoid binders characteristics of Type IB receptors (i.e., the receptors did not react with a monoclonal antibody raised against Type II receptors and they were not retained by DEAE-cellulose columns). In contrast, Goldman and Katsumata (1986) have reported that Type IB receptors could be detected in lung cytosol only when cytosol was prepared in hypotonic buffers containing molybdate. Although the questions as to whether Type IB receptors exist in vivo and whether they have physiological significance remain to be established, it is entirely possible that these receptors may be generated from Type II receptors by specific proteases in target tissues that appear to contain these receptors.

Experiments with adrenalectomized animals treated with amiloride prior to injection of ALDO suggest that the increase in Na-K-ATPase activity may be a secondary adaptation to sodium transport induced by this steroid (Doucet and Katz, 1981b; Petty et al., 1981). Furthermore, at least in toad urinary bladder, it appears that the stimulatory action of ALDO on sodium transport is not mediated by the synthesis of new (Geering et al., 1982, Park and Edelman, 1984b) or activation of the pre-existing sodium pumps (Park and Edelman, 1984b).

The idea that AIPs may be a component of the plasma membrane (on the luminal, urine, surface) of the cell, where they act as a sodium permease to lower the resistance to Na^+ entry into the cell, was proposed originally by Crabbe and de Weer (1965) and Sharp et al. (1966a) and subsequently gained experimental support through work in the toad urinary bladder (Civan and Hoffman, 1971; Spooner and Edelman, 1975; Palmer et al., 1982; Park and Edelman, 1984a). All of these

studies, however, fail to demonstrate whether AIPs act as permeases themselves, or whether they modulate the conformation or arrangement of pre-existing permease molecules in the membrane such that they are more accessible for Na^+ entry. A recent study by Kipnowski et al. (1983) found that ALDO fails to increase sodium transport in tissues where transport was inhibited by N-ethoxycarbonyl-2-ethoxy-1,2-dihydro-quinoline (EEDQ) prior to exposure to this steroid. In contrast to results with cycloheximide (an inhibitor of protein synthesis), EEDQ did alter ALDO stimulation of the osmotic water flow in response to antidiuretic hormone. These data do not therefore support the notion that AIPs are new sodium channels, but instead they are consistent with the hormonal activation of pre-existing, nonfunctional channels.

The first evidence that mineralocorticoids may induce the synthesis of a mitochondrial enzyme participating in tricarboxylic acid cycle was obtained by Kirsten et al. (1968) who found that ALDO increased the citrate synthase, isocitrate dehydrogenase, glutamate dehydrogenase, glutamate-oxaloacetyl transaminase and malate dehydrogenase activity in the toad bladder. The increase in the synthesis of mitochondrial enzymes was postulated to reflect hormone-controlled mudulation of the available ratio of ATP/ADP which, in turn, could alter both Na^+ conductance and active transport. ALDO has also been shown to increase the ratio of $NADH/NAD^+$ in adrenalectomized rat kidneys (Kirsten and Kirsten, 1972). Similarly, Law and Edelman (1978b) found an increase in renal cortical and medullary citrate synthase activity three hours after administration of ALDO to adrenalectomized rats. In this latter study ALDO was found to enhance incorporation of radiolabeled methionine into renal, but not hepatic, citrate synthase. The relevance of these

observations with reference to the energy theory of mineralocorticoid action lies in the fact that actinomycin D and specific spironolactones inhibit the ALDO-induced increase in mitochondrial enzymatic activities, and that DEX has no effect on the hormonal induction (Law and Edelman, 1978b). Furthermore, this action of ALDO appears to be target specific: ALDO had no detectable effect on the citrate synthase activity in the renal papilla or the liver under the same conditions (Law and Edelman, 1978b).

In summary, although much has been done to expand our knowledge of the molecular mechanisms of mineralocorticoid action, the exact mechanism by which these hormones exert their effects still awaits further exploration. It is, nevertheless, clear that the functional consequences of mineralocorticoid action involves, at least in part, the genetic machinery in target cells and an ultimate modulation of the synthesis and/or activation of the pre-existing structural and regulatory proteins.

Mechanisms of Receptor Regulation

There is considerable evidence to suggest that the in vivo binding capacity of adrenocorticosteroid hormone receptors is subject to change in response to a variety of intra- and extracellular signals (McEwen, 1979; Muldoon, 1980; Svec, 1985b; De Kloet et al., 1986; McEwen et al., 1986a). For example, hyperkalemic adrenalectomized rats were shown to have an elevated renal cytosolic Type I receptor binding capacity (Rafestin-Oblin et al., 1984). Similarly, adrenalectomy (adrenocorticosteroid deprivation) alone was found to result in a time-dependent increase in the maximal binding capacity of both Type I and Type II receptors in all tissues investigated (Beato et al., 1974;

McEwen et al., 1974; Gregory et al., 1976; Ichii, 1981; Claire et al., 1981; Muramatsu et al., 1983, Rafestin-Oblin et al., 1984; Turner, 1986; Sarrieau, et al., 1986; Luttge and Rupp, unpublished). It is noteworthy that adrenalectomy was found to induce a differential tissue-specific up-regulation in Type I and Type II receptor binding capacity in mice. In the three tissues investigated (i.e.; brain, liver and kidney), adrenalectomy was found to induce the greatest up-regulation in the brain cytosolic Type I receptors -- a 10- to 14-fold rise compared to the adrenally intact control group as measured by saturation and equilibrium binding analyses (Luttge and Rupp, unpublished). Adrenalectomy also induced a similar tissue-specific up-regulation in Type II receptors: The largest increase was found in the kidney cytosol, followed by amygdala-entorhinal cortex, hippocampus, liver, cerebral cortex, hypothalamus, pituitary and heart (Turner, 1986).

In Scatchard plots (Scatchard, 1949), an increase in the B_{MAX} as a mode of "up-regulation" of Type I and Type II receptors induced by adrenalectomy is a common finding (Beato et al., 1974; McEwen et al., 1974; Gregory et al., 1976; Claire et al., 1981; Ichii, 1981; Tornello et al., 1982; Muramatsu et al., 1983; Rafestin-Oblin et al., 1984; Sapolsky et al., 1984; Luttge and Rupp, unpublished). This increase in the B_{MAX} has been postulated to reflect an unmasking of receptors previously occupied by endogenous ligands (Feldman, 1974; McEwen et al., 1974; Giannopoulos, 1975), "activation" of an already available pool of receptors to a ligand-binding state, an increase in the rate of receptor synthesis (McEwen et al., 1974; Claire et al., 1981) and/or a reduction in the rate of receptor degradation (Claire et al., 1981).

Treatment of adrenalectomized animals with CORT, ALDO and other steroid agonists that show appreciable affinity for Type I and Type II receptor binding sites results in a reduction in the ligand binding capacity of these receptors (Grekin and Sider, 1981; Ichii, 1981; Tornello et al., 1982; Muramatsu et al., 1983; Rafestin-Oblin et al., 1984; Sarrieau et al., 1986; Luttge and Rupp, unpublished). Repeated stress (a condition that induces the production of high circulating levels of CORT) was also found to result in a significant reduction in Type II receptor binding capacity in the rat hippocampus (Valeri et al., 1978; Sapolsky et al., 1983; Sapolsky et al., 1984; Sapolsky, 1985; Sapolsky et al., 1985b) and amygdala (Sapolsky et al., 1983; Sapolsky et al., 1984). In addition to regulation of their own receptors, chronic administration of stress level concentrations of corticosteroids was found to have neurotoxic effects on hippocampal neurons (Sapolsky, 1985; Sapolsky et al., 1985b). Similarly, in cultured cell lines (HeLa S3, AtT-20 and GH_1 cells), glucocorticoids were shown to "down-regulate" their own receptors (Cidlowski and Cidlowski, 1981; Svec and Rudis, 1981; Raaka and Samuels, 1983; Svec, 1985a). This down-regulatory effect of ligand on its own receptor has been reported for a number of other receptors; e.g., progesterone receptors (Milgrom et al., 1973b; Walters and Clark, 1979), thyroid hormone receptors (Raaka and Samuels, 1981), insulin receptors (Gavin et al., 1974; Huang and Cuatrecasas, 1975; Olefsky, 1976) and other peptide receptors (Catt et al., 1979; Roth and Taylor, 1982).

In GH_1 cells, incubation with triamcinolone acetonide (TA) was found by McIntyre and Samuels (1985) to reduce the half-life of Type II receptors by more than 50% (from 19.0 to 9.0 h) as determined through

the use of dense amino acid labeling of the receptors. Consistent with this finding, in the presence of TA, the levels of Type II receptors in these cells were half that measured in the control cells (i.e., about 130 vs. 260 fmole/100 ug DNA, respectively). The addition of TA to the culture media had little effect on the rate of de novo receptor synthesis. It was shown that TA promoted the activation of Type II receptors (transformation to a state with increased affinity for DNA, see Chapter VI), and that the activated form was degraded at a faster rate (than the unactivated form) thus accounting for the reduction in the receptor half-life (McIntyre and Samuels, 1985). This apparent faster rate of degradation may represent the inability of the activated receptors to rebind ligand, since recent work from our laboratory has shown that following heat-induced transformation to a DNA-binding form the activated receptors do not rebind ligand after dissociation (Chou and Luttge, 1987). Recently, treatment of rat hepatoma culture cells with DEX was shown to result in an initial increase (after 6 h), followed by a reversible (after 72 h) 50% to 95% decrease in Type II receptor mRNA (after 24 h) in these cells (Okret et al., 1986). In this latter study, using an immunoprecipitation assay, Type II receptors were shown to interact specifically with a Type II receptor cDNA clone, hence providing a more direct evidence for the ligand-induced receptor autoregulation (Okret et al., 1986).

The up- and down-regulation of the binding capacity of receptors for adrenal steroids in the central nervous system raises the possibility that receptor autoregulation, and hence modulation of brain sensitivity to steroids, may ultimately be involved in such functional consequences as the feed-back control mechanisms of adrenal steroid

secretion (Cake and Litwack, 1975; Sapolsky et al., 1985a; for review see Sapolsky et al., 1986).

A second mode of receptor regulation, at least in adrenocorticosteroid Type II and progesterone receptor systems, may involve a rapid turning "on" and "off" of the unoccupied receptors in response to different stimuli. This rapid and reversible loss of steroid binding capacity has been shown to involve a phosphorylation-dephosphorylation cycle of the receptors in vitro and possibly in vivo (for review see Housley et al., 1984). In an earlier report, Munck and co-workers (1972) proposed that Type II receptors in thymocytes exist in two states; the steroid-binding form and those that did not bind steroids. It was proposed that ATP was required to convert the non-binding receptors to a state that bound steroid (Munck et al., 1972). Consistent with this finding, receptor stability was reported to be enhanced by including ATP and other nucleotides (a requirement for the action of some phosphorylase enzymes) in cytosol preparations (Nielsen et al., 1977b; Sando et al., 1979a & b). Our laboratory has recently shown that exogenous ATP can also prevent the destabilizing actions of as yet unknown microsomal components on Type II receptors (Densmore and Luttge, 1985). The use of phosphatase inhibitors (e.g., fluoride and molybdate) in cytosol preparations has been shown to improve considerably the stability of Type II receptors (Nielsen et al., 1977b). Conversely, the addition of highly purified calf intestine alkaline phosphatase to cytosol preparations markedly destabilized these receptors (Nielsen et al., 1977a). A striking, positive correlation between ATP concentration and Type II receptor binding capacity in a human cell line (i.e., IM-9 cells) was also observed (Wheeler et al.,

1981). Lastly, affinity labeling of Type II receptors (from L-cells grown in the presence of [^{32}P]orthophosphate) with dexamethasone 21-mesylate has provided direct evidence suggesting that these receptors may be phosphoproteins (Housley and Pratt, 1983). It is important to note that although these data clearly favor the role of phosphorylation-dephosphorylation as a possible mechanism of Type II receptor regulation, there is as yet no experimental evidence to suggest a similar mechanism for up- and down-regulation of Type I receptors.

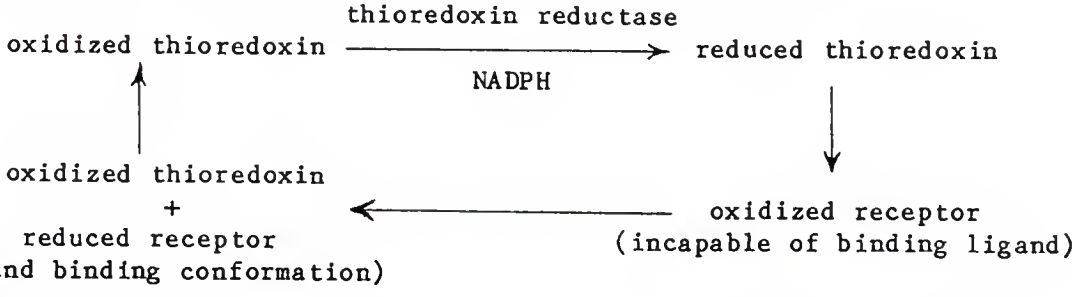
A third mechanism for the potential regulation of Type II and possibly Type I receptors (which may also be related to the phosphorylation-dephosphorylation cycle (Housley et al., 1982, for review see Housley et al., 1984)), is the reduction and oxidation of sulfhydryl group(s) in the receptor macromolecule. It is well known that thiol-disulfide exchanges can markedly affect the activity of a number of protein macromolecules including androgen, estrogen, progesterone, ecdysone and insulin receptors, lysozyme, immunoglobulins and many digestive enzymes. In 1975, Rees and Bell claimed that they were able to recover partially the loss in binding capacity of rat thymic cytosol receptors by including sulfhydryl reducing reagents, e.g.; dithiothreitol (DTT) and 2-mercaptoethanol, in their buffers. Two years later, Granberg and Ballard (1977) reported that the addition of 2 mM DTT to rat lung cytosol increased the apparent binding of Type II receptors 10-fold. This increase was proposed to be due to transformation of an already-available pool of receptors to a ligand-binding state. Similar effects were also seen in brain, uterus, thymus and a number of other tissues whereas the presence or absence of DTT was found to have little effect on Type II receptor binding capacity

in liver, kidney and heart cytosol. It was postulated that these latter tissues contained endogenous, heat-stable, sulfhydryl reducing agents which stabilize Type II receptor binding capacity. Interestingly, an analysis of Type II receptors in fetal (Ballard et al., 1974) and adult (Granberg and Ballard, 1977) rat lung revealed that exogenous DTT was required only in the cytosol prepared from adults. Together these observations suggest that variations in the production of sulfhydryl reducing (and/or oxidizing) reagents may be responsible for differential stabilization of Type II receptors in various tissues and in the same tissue during different stages of development.

An endogenous protein molecule, capable of modulating steroid binding capacity of the rat liver cytosolic unoccupied Type II receptors, has recently been characterized by Pratt and co-workers (Grippo et al., 1983 & 1985). This endogenous receptor-reducing agent appears to be an NADPH-dependent thioredoxin. Extraction of this stabilizing factor from liver cytosol by charcoal resulted in a significant loss of unoccupied, but not occupied Type II receptor binding capacity. Addition of DTT to the charcoal-extracted cytosol resulted in a complete recovery of Type II receptor binding capacity (Grippo et al., 1983). Similar results have been obtained in our laboratory. We found that charcoal extraction of steroid-free brain cytosol prepared in HEPES [4(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer containing 10% glycerol and 2 mM DTT resulted in an increase in the thermal instability and a decrease in the total binding capacity of Type II receptors (Emadian et al., 1986). These effects were reversible after the readdition of the exogenous DTT removed by the charcoal treatment. In further investigating the effect of the presence

or absence of DTT, we found that the omission or removal of this sulfhydryl reducing reagent produced a significant increase in the apparent K_d and a slight reduction in the B_{MAX} of [^{3}H]DEX binding to Type II receptors (Densmore et al., 1984b).

The effect of DTT on Type II receptor binding capacity appears to be a temperature-independent phenomenon (Rees and Bell, 1975; Granberg and Ballard, 1977; Grippo et al., 1983; Densmore et al., 1984; Emadian et al., 1986), whereas the NADPH-dependent thioredoxin appears to act in a temperature-dependent fashion (Grippo et al., 1983). These findings suggest that the exogenous sulfhydryl reducing reagents may act primarily on the receptor [or other component(s) of the system] to maintain it in a reduced state -- the conformation necessary for steroid binding. In contrast, the reducing effects of thioredoxin must be mediated primarily through an enzymatic process (Grippo et al., 1983 & 1985). Thioredoxin reductase was found to be responsible for the transfer of reducing equivalents from NADPH to thioredoxin. The reduced form of the thioredoxin, in turn, is thought to maintain the receptors in the steroid-binding state (Grippo et al., 1985). The transfer of the reducing equivalent may be shown schematically as follows:



The observation that occupied receptors from charcoal extracted cytosol (i.e., in the absence of thioredoxin and/or DTT) appear to be unaffected by charcoal treatment has raised the possiblity that the

sulfhydryl group(s) on the receptor may be located at or near the steroid binding domain (Grippo et al., 1983). Alternatively, the binding of ligand to Type II receptor could induce a conformational change in the receptor molecule which renders more distant sulfhydryl group(s) inaccessible for oxidation. With regards to this latter hypothesis, a recent preliminary study from our laboratory suggests that DTT may have no detectable effects on the sedimentation coefficient of unoccupied Type II receptors (Densmore et al., 1984). It is important to remember that the method of sucrose density gradient ultracentrifugation may not be sensitive enough to reveal small conformational changes in receptor.

Another possible regulatory role of the thiol-disulfide exchange mechanism involves the interaction of steroid-receptor complexes with the cellular genomic machinery. Preatreatment of DEX-receptor complexes with sulfhydryl oxidizing reagents [e.g.; methyl methanethiosulfonate, MMTS and 5,5'-dithiobis (2-nitobenzoic acid), DTNB] inhibits subsequent binding of the activated receptor complexes to DNA-cellulose (an _in vitro_ index for receptor transformation) (Bodwell et al., 1984). Interestingly, the inhibitory effect of these reagents can be reversed by the addition of DTT. These results were interpreted to suggest that sulfhydryl group(s), located at or near DNA-binding domain of the activated steroid-receptor complex, may regulate the interaction of the complex with the genome (Bodwell et al., 1984). It is noteworthy that although MMTS and DTNB were shown to diminish DNA-cellulose binding of the complexes, the authors (Bodwell et al., 1984) failed to refute conclusively the possibility that these reagents inhibited DNA-binding by acting as cross-linking reagents (i.e., cross-linking of the

activated receptors with other cytosolic components such as heat-shock proteins, mRNAs, etc.). In fact, current data clearly favors the notion that N-ethylmaleimide (NEM) and iodoacetamide (other sulfhydryl modifying reagents) inhibit transformation of rat liver Type II receptors (Kalimi and Love, 1980) probably through inhibition of subunit dissociation (for detail see Chapter VI). Furthermore, it is possible that the bulky thiomethyl and thionitrobenzoate complexes (formed by the interaction of MMTS and DTNB with the receptors, respecively), rather than the oxidation itself, inhibit the binding of the complexes to DNA-cellulose. More recently, Type II receptors were shown to bind nuclear matrix (a proteinaceous structural framework implicated in many nuclear functions, for detail see Chapter VI) through intermolecular disulfide bond formation (Kaufmann et al., 1986). Sodium dodocylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the rat hepatic Type II receptors extracted from nuclei isolated from animals injected with [^{3}H]TA in the absence of sulfhydryl-blocking (e.g., iodoacetamide) and cross-linking reagents yielded steroid-receptor complexes that were disulfide-cross-linked to the nuclear matrix. Conversly, isolation of the nuclei in presence of iodoacetamide, revealed no receptor-nuclear matrix complex formation (Kaufmann et al., 1986). These results provide a more direct evidence for the involvment of sulfydryl groups in the binding of transformed steroid-receptor complexes to nuclear components (for further discussion see Chapter VI). Recently, structural analysis of the amino acid sequences of a number of steroid receptors revealed the presence of a cysteine rich region in the DNA-binding domain these receptors (e.g., Danielsen et al., 1987).

In contrast to our understanding of the factors regulating Type II receptor binding activity, our knowledge concerning possible up- and down-regulatory factors affecting Type I receptors remains scanty. As discussed above, we have demonstrated several *in vitro* chemical differences between the brain cytosolic Type I and Type II receptors which may play an important role in the *in vivo* regulation of these receptors. For example, although charcoal-extraction of steroid-free brain cytosol in HEPES buffer lacking DTT causes a dramatic reduction in Type II binding capacity (possibly by removal of endogenous thioredoxin), this treatment has no effect on the binding capacity of Type I receptors (Emadian et al., 1986; also see Chapter V). This finding suggests that the labile thiol-disulfide exchange mechanism described for Type II receptors may not be involved in the regulation of Type I receptor binding capacity. In an effort to shed more light on the role of possible factors and mechanisms involved in the *in vitro* and/or *in vivo* regulation of Type I receptors, an investigation of possible involvement of sulfhydryl groups in the ligand- and DNA-binding capacity of these receptors was conducted in this dissertation (see Chapters V and VI).

In Vitro Analysis of Type I Receptors by Radioreceptor Assay

A clear understanding of the physiological and behavioral consequences of steroid hormone action requires an unambiguous characterization of the receptors through which such actions presumably are mediated. Our knowledge of the properties of Type I receptors as well as the factors involved in the regulation of these receptors may indeed lead to further discoveries regarding functional consequences of these steroids. For example, the recognition of a specific

mineralocorticoid receptor led to the discovery of spironolactones (mineralocorticoid antagonists), which have proven invaluable tools clinically (as effective antihypertensive agents) and in basic sciences (see above). Although numerous studies investigating the properties of steroid hormone receptors have appeared in the literature within the past two decades, the volume of research on the properties of Type I receptors has been comparatively minimal (Liao et al., 1983). This is partially due to the fact that in crude preparations ALDO and other readily available synthetic mineralocorticoids bind to Type II receptors with appreciable affinity (Rousseau et al., 1972; Funder et al., 1973a; Emadian et al., 1986; Emadian and Luttge, unpublished; also see Chapter V). This indiscriminatory binding of ALDO to Type II receptors can produce confounding results thus hampering an independent analysis of Type I receptors. Although the use of mathematical models has proven effective in determination of binding parameters for Type I receptors (e.g., Claire et al., 1978; Claire et al., 1985; Emadian et al., 1986), failure to eliminate Type II-binding interferences has led a number of investigators reach erroneous conclusions in their analysis of Type I receptor properties (Grekin and Sider, 1980; Marver et al., 1972). Recently, however, the introduction of very specific synthetic Type II ligands (e.g., RU 26988, RU 28362 and RU 38486) has made the elimination of Type II-binding contribution in crude cytosolic preparations possible. When used in conjunction with radiolabeled ALDO, a molar excess of the RU compound can saturate all Type II binding sites with little or no cross-reactivity with Type I receptors, hence leaving these latter sites intact for ALDO binding. This method has been utilized successfully by this and a number of other laboratories (Moguilewsky and

Raynaud, 1980; Veldhuis et al., 1982b; Gomez-Sanchez and Gomez-Sanchez, 1983; Wrange and Yu, 1983; Emadian, et al., 1986).

In addition to these methods, Veldhuis et al. (1982b) reported that they were able to eliminate non-linearities observed in the Scatchard plots for ALDO binding by co-incubating hippocampal cytosol with unlabeled CORT. Although the Scatchard plot of ALDO binding in the presence of a 100-fold concentration of RU 26988 appears comparable to that obtained in the presence of a 0.6-fold concentration of CORT (Veldhuis et al., 1982b), in light of the fact that CORT has a higher affinity for hippocampal Type I receptors (see below), a lower value of equilibrium maximal binding in the presence of CORT is consistent with the interaction of CORT with Type I receptors (for detail see below). To eliminate the confounding effects of ALDO interaction with Type II receptors, unless otherwise stated, the characterization of Type I receptors in this dissertation was accomplished in the presence of a 500-fold molar excess of RU 26988, a concentration determined to eliminate all Type II binding interferences (Emadian et al., 1986) (for detail see Chapter II).

Another pertinent issue in the in vitro analysis of steroid receptors in crude cytosolic preparations, in general, is possible contamination and thus binding contribution by low affinity and high capacity "non-specific binders" (of cytosolic, membrane or of plasma origin). It is well established that ALDO binds with appreciable affinity, especially at lower tempertures, to corticosteroid binding globulin (CBG) (Sandberg et al., 1960; Daughaday et al., 1960; Meyer et al., 1961; Davidson et al., 1962; Zager et al., 1976; Zipser et al., 1980), and with a lower affinity to α_1-acid glycoprotein and albumin

(Chen et al., 1961; Daughaday et al., 1960; Ganguly and Westphal, 1968). Moreover, using competitive adsorption and gel filtration techniques, Richardson and co-workers (1977) identified two other plasma ALDO-binding proteins distinct from CBG, albumin and α_1-acid glycoprotein in human plasma. This has been confirmed by Katayama and Yamaji (1982) who reported that this "ALDO binder" had an apparent association constant for ALDO one order of magnitude higher than that for CBG. Although there is no evidence that these plasma binders are present in rodents, because 1) ligand binding assays are usually performed at lower temperatures and 2) corticosteroid-binding globulin (CBG) shows appreciable affinity for ALDO at lower tempertures, an extensive in situ transcardiac perfusion of brains prior to cytosol preparation will be performed. This will assure the elimination of possible contribution of these binders in our in vitro characterization of Type I receptors. Note that this perfusion was deemed sufficient in eliminating these binders, since co-incubation of whole brain cytosol with [^{3}H]ALDO and cortiuic acid (a synthetic 17α-acid derivitive of cortisol that specifically binds CBG, see Alexis et al., 1983; Little, 1983; Sheppard and Funder, 1986) produced specific Type I receptor binding similar in magnitude to that found in the presence of [^{3}H]ADLO alone.

In addition to the above plasma binders, the presence of the extravascular corticosterone binder (with identical physicochemical and immunoreactive properties to the plasma CBG) has been identified in the uterus (Milgrom and Baulieu, 1970; Guerigian et al., 1974; Al-Khouri and Greenstein, 1980), liver (Koblinsky et al., 1972; Litwack et al., 1973; Amaral et al., 1974; Suyemitsu and Terayama, 1975; Weiser et al., 1979),

kidney (Funder et al., 1973b; Feldman et al., 1973; Strum et al., 1975; Weiser et al., 1979), pituitary and brain (Koch et al., 1976; De Kloet and McEwen, 1976a & b; Al-Khouri and Greenstein, 1980; Gray and Luttge, 1982), and a number of other tissues (Mayer et al., 1975; Giannopoulos, 1976; Werthamer et al., 1973) (for review see Siiteri et al., 1982). The order of steroid binding specificity for these CBG-like binders determined by competition assays appears to be CORT > DOC >> ALDO >> DEX. Although it has been shown conclusively that the synthesis of CBG-like glucocorticoid binders (by convention, referred to as Type III receptors) occurs in the liver (Perrot-Applanat and Milgrom, 1979; Weiser et al., 1979), the possible functional roles and the origin of these binders in other tissues have been the subject of considerable controversy. Since a comprehensive review of the primary references surrounding this issue is outside the scope of this dissertation, only the recent findings relevant to the in vitro characterization of Type I receptors will be reviewed.

More than a decade ago, it was established that in kidney there were two distinct classes of corticosteroid binding proteins representing the Type I and Type II receptors (Funder et al., 1973b). It was further claimed that the hierarchy of steroid binding specificity for Type I receptors were ALDO > DOC > CORT (Funder et al., 1973b). Later, the presence of specific Type I receptors in a number of other tissues including the CNS were reported (Anderson and Fanestil, 1976; Moguilewsky and Raynaud, 1980; Lan et al., 1981). However, it appeared that ALDO, DOC and CORT had equivalent affinity for these sites. The results from these in vitro competition assays were taken as evidence for possible in vivo occupancy of Type I receptors by

glucocorticoids in the rat kidney and brain (Lan et al., 1982; Veldhuis et al., 1982b). In a separate study, Beaumont and Fanestil (1983) reported that the steroid binding specificity Type I receptors in rat brain could be altered by different experimental manipulations. These investigators found that after elimination of Type II binding interferences (in the presence of a molar excess of RU 26988) and plasma CBG (and possibly Type III receptors), the order of steroid competition for Type I sites (occupied with [^{3}H]ALDO) was DOC > fludrocortisone > CORT > ALDO > progesterone > DEX. However, the addition of small quantities of dialyzed serum to the above preparations yielded a competition hierarchy similar to that described for kidney, i.e., ALDO > DOC > CORT. Several independent confirmations of the above observations were reported for the rat kidney (Krozowski and Funder, 1983; Stephenson et al., 1984b) and hippocampus (Krozowski and Funder, 1983; De Kloet et al., 1984). In conclusion, it is suggested that CBG and CBG-like binders, in addition to affecting the metabolic clearance rate of the steriods to which they bind with high affinity (for review see Siiteri et al., 1982), may act in vivo as sequestration sites or "sinks" for corticosterone thus rendering the Type I sites available for ALDO binding (Funder et al., 1973b; Krozowski and Funder, 1983; Stephenson et al., 1984; Funder, 1986). It is further suggested that the hippocampal Type I receptors are physiological CORT binders and may be involved in a tonic (permissive) influence on brain function with septo-hippocampal complex as a primary target (De Kloet et al., 1984; Reul and De Kloet, 1985; Funder, 1986; Magarinos et al., 1986; Reul and De Kloet, 1986). Although the sequestration theory is an attractive hypothesis, further experiments investigating the tissue availability of CORT (that is not

bound to CBG, other plasma binders and Type III receptors) and ALDO under physiological conditions is required to establish this theory. In this regard, preliminary studies suggest that the serum levels of ALDO and CORT under "normal" conditions leads to nuclear uptake of both hormones in the hippocampus as measured by radioimmunoassay (Yongue and Roy, 1984). Recently, McEwen et al. (1986b) showed that, in adrenalectomized rats, only "stress level" concentrations (a 500-fold molar excess) of CORT was able to block the nuclear uptake of [^{3}H]ALDO in limbic, but not circumventricular, areas. Further functional relevence of these findings remain to be established.

A further caution regarding the results obtained from in vitro competition assays also deserves mention: in the above experiments cytosolic preparations were performed in the presence of molybdate and DTT. Our own work has shown that although molybdate stabilizes both Type I and Type II receptors, it causes a significant reduction in the binding capacity of Type I receptors (Emadian et al., 1986; also see Chapter IV). Furthermore, sulfhydryl reducing reagents such as DTT, while having no effect on the binding of ALDO to Type I receptors, result in an apparent increase in the equilibrium dissociation constant of CORT from CBG (Westphal, 1983) and very likely from Type III receptors. Therefore, under conditions that provide optimal stability for all of these proteins, one may find different steroid-binding hierarchy from those discussed above.

CHAPTER II
EXPERIMENTAL PROCEDURES

In order to avoid repetition, in this chapter a general scheme of the materials and experimental procedures shared by the studies described in Chapters III through VI will be presented. Materials and methods that are unique to a given chapter will be described in that particular chapter.

Isotopes, Steroids and Other Chemicals

The radiolabeled steroids; i.e., [6,7-^{3}H]Triamcinolone acetonide, [^{3}H]TA, 9α-fluoro-11β, 16α,17,21-tetra-ol-pregna-1,4-diene-3,20-dione (specific activity, SA 43.7 Ci/mmol); [6,7-^{3}H]dexamethasone, [^{3}H]DEX, 9α-fluoro-16α-methylprednisolone (SA 37.3-44.1 Ci/mmol) and [1,2,6,7-^{3}H]aldosterone, [^{3}H]ALDO, 4-pregnen-11β,21-diol-3,18, 20-trione (SA = 71.5-82.0 Ci/mmol) were purchased from New England Nuclear. These radiolabeled steroids were checked for purity by thin-layer silica gel chromatography using a chloroform-methanol (9:1, volume:volume) solvent system. [^{1}H]TA, [^{1}H]DEX, [^{1}H]ALDO, Sephadex G-25 (bead size 20-80 u) and sodium molybdate were obtained from Sigma. HEPES buffer [4(2-hydroxyethyl)-1-peperazineethanesulfonic acid] and dithiothreitol (DTT) were obtained from Research Organic. RU 26988 [11β,17β-dihydroxy-17α-(1-propionyl)-androsta-1,4,6-triene-3-one)] and RU 26752 [3' (3-oxo-7α-propyl-17β-hydroxy-4-androstene) propionic acid lactone] were kindly supplied by Roussel-Uclaf (France). Prorenone

[3(17β-hydroxy-6β,7β-methylene-3-oxo-4-androsten-17α-yl) propionic acid γ-lactone] was a gift from Searle Co. All other chemicals used in this dissertation were reagent grade quality.

Experimental Animals

Adult female CD-1 mice (Charles River Laboratories) were subjected to a combined bilateral adrenalectomy and ovariectomy under barbiturate anesthesia 3-6 days prior to use [a period shown to result in maximal up-regulation of Type I receptors (Luttge and Rupp, unpublished results)] in order to remove known sources of endogenous steroids. They were then maintained on Purina Rodent Chow and 0.9% NaCl. On the day of experiment, mice were anesthetized with ether and perfused slowly through the heart with 15-20 ml ice-cold HEPES-buffered saline (isotonic, pH 7.60).

Table 2-1

Buffers

BUFFER	20 mM HEPES	2 mM DTT	Na_2MoO_4
A	+	-	-
D	+	+	-
M	+	-	2 mM
M_{20}	+	-	20 mM
DM	+	+	2 mM
DM_{20}	+	+	20 mM

The cytosolic concentration of the component(s) in Buffers D, M, M_{20}, DM, DM_{20} as well as other cytosolic components (e.g., 300 mM KCl; 10% mono- and polyhydric compounds) were obtained by a 9:1

dilution of cytosol with stock buffers containing a 10-fold concentration of the desired component(s). Under such circumstances, the control group was diluted accordingly with an equivalent volume of Buffer A. Buffers were adjusted to pH 7.60 at 0-2°C.

Cytosol Preparation

Brains were homogenized (2 x 10 strokes) at 1000 RPM in two volumes of ice-cold Buffer A or Buffer A containing one or more of the desired components (see specific experiments). A glass homogenizer with a teflon pestle milled to a clearance of 0.125 mm on the radius was used to minimize the rupture of brain cell nuclei. The crude homogenate was centrifuged at 100K x g for 20 min and the resulting supernatant recentrifuged at 100K x g for an additional 60 min to yield cytosol. During this and all other procedures (except "aging" and column chromatography) attempts were made to maintain the cytosol at 0°C.

Aging

In order to examine thermal stability of unoccupied Type I and Type II receptors, cytosol was incubated for various periods of time at 0 and/or 22°C. Following such treatment, "aging" was quenched by incubating the cytosol with steroid(s) for 24-48 h at 0°C for specific binding determination (see below).

Hydrophobic Interaction Chromatography.

Pently-Agarose columns (0.7 x 21.5 cm) were used to compare the surface hydrophobic properties of occupied Type I versus Type II (Chapter III) as well as activated versus unactivated Type I receptors (Chapter VI). In such experiments, cytosol in Buffer DM_{20} was incubated with radiolabeled ligand for Type I or Type II receptors for 48 h at 0°C (see "Steroid Binding Determination"). Following incubation

with steroid(s), salt- and heat-induced activation (see Chapter VI), bound-free separation on Sephadex G-25 (0.6 x 14.0 cm) columns were performed as described previously (Luttge et al., 1984c). Subsequently, 0.8 ml [^{3}H]steroid-bound macromolecular fraction eluted from the G-25 column was loaded on pently-Agarose columns pre-equilibrated in buffer D containing 50 mM molybdate and 600 mM KCl. Using this latter buffer, 20-27 consecutive 0.8 ml fractions were collected directly into scintillation vials for measurements of radioactivity (see below). In some experiments, a second bound-free separation on G-25 columns were performed on the "peak" fractions eluted from the pentyl agarose columns to assure that the radioactivity eluted was indeed macromolecular-bound in nature. All chromatographic procedures were performed at 4-6°C.

Steroid Binding Determination

After appropriate treatment of cytosol, 0.24-4.0 ml aliquots were added to pre-cooled glass tubes containing the desired steroid(s) (dried under N_2). The tubes were then allowed to incubate for 24-48 h in ice. In single saturation dose studies, B_T determinations for Type I receptors were performed in the presence of 10-20 nM [^{3}H]ALDO and 500-fold molar excess of RU 26988. For Type II receptors, 20 nM [^{3}H]DEX or [^{3}H]TA was used as the ligand. In some experiments, [^{3}H]DEX incubation was in the presence of a 500-fold molar excess of RU 26752, prorenone or RU 26988 (see Chapter V). Parallel incubation tubes containing an additional 200-fold molar excess of [^{1}H]ALDO, [^{1}H]DEX or [^{1}H]TA were used for the determination of B_{NS} for Type I and Type II receptors, respectively. Equilibrium binding parameters; i.e., K_d and B_{MAX} were determined using Scatchard's method (1949) as described previously (Emadian et al., 1986).

In all experiments, macromolecular-bound from free steroid was separated at 4°C using G-25 (0.6 x 14 cm) columns (for detail see Luttge et al., 1984c). Bound fraction (0.8 ml) obtained from each column was diluted with 5 ml Scinti-Verse fluor (Fisher Scientific) in a scitillation vial, vortexed vigorously and the radioactivity determined in a Packard Model 2425 liquid Scintillation spectrometer. All counts per minute (CPM) data were converted to disintegration per minute (DPM) by an external standard channels ratio measurement. The counting efficiency for tritium averaged about 35%.

Cytosolic Protein Concentration

The concentration of protein in cytosol preparations was determined using the method described by Lowry et al. (1951). In crude preparations, protein concentration ranged from 6.2 to 7.3 mg/ml cytosol, whereas the values in gel filtered and 2-fold diluted preparations (see Chapter IV) averaged about 3.3 and 3.6 mg/ml cytosol, respectively.

CHAPTER III

EFFECTS OF POLYHYDRIC AND MONOHYDRIC COMPOUNDS ON THE STABILITY OF TYPE I RECEPTORS FOR ADRENOCORTICOSTEROID HORMONES IN BRAIN CYTOSOL

Summary

We have shown previously that unoccupied Type I receptors for adrenal steroids in brain cytosol lose their ability to bind [^{3}H]ALDO in a time- and temperature-dependent manner (Emadian et al., 1986). Based on reports that sugars and polyvalent alcohols are capable of stabilizing a variety of globular proteins, we made an attempt in this study to stabilize Type I receptors by including polyhydric compounds in our cytosol preparations. However, unlike our expectations, the addition of 10% (weight:volume) ethylene glycol, glycerol, erythritol, xylitol, ribitol or sorbitol to cytosol at 0°C failed to stabilize the receptors and in fact produced a slight reduction in the binding of [^{3}H]ALDO. The magnitude of this reduction was greater when cytosol was "aged" for 2 h at 22°C prior to incubation with [^{3}H]ALDO. In contrast to these results with polyhydric compounds, the addition of 10% (weight:volume) ethanol to cytosol maintained at 0°C markedly increased the binding of [^{3}H]ALDO to Type I receptors. Under identical conditions, methanol slightly increased, and propanol had no significant effect on the binding capacity of these receptors. When cytosol was aged at 22°C, however, all of these monohydric compounds led to a marked loss in Type I receptor binding capacity. An investigation of various doses of ethanol at 0°C on the subsequent binding of [^{3}H]ALDO to Type I receptors

yielded an inverse U-shaped curve with 10% ethanol producing the highest level of specific binding, as reflected by an increase in maximal binding in Scatchard plots, and 40% ethanol producing a complete loss in Type I receptor binding capacity. In view of the above findings and proposed mechanisms of action of mono- and polyhydric compounds, it is suggested that Type I receptors in an aqueous cytosolic environment undergo spontaneous conformational changes which promote the infolding of hydrophobic steroid binding site(s) on the receptor surface, thus leading to the observed reduction in Type I receptor binding capacity.

Introduction

Although receptors for steroid hormones appear to be relatively stable under in vivo conditions (Raaka and Samuels, 1983), these macromolecules are rendered labile under cell-free conditions. This increased lability is particularly apparent when working with unoccupied receptors (Puca et al., 1971; Pratt and Ishii, 1972; Bell and Munck, 1973; Rafestin-Oblin et al., 1977; Emadian et al., 1986), and it can be a serious problem when experimental conditions necessitate that the unoccupied receptors must remain stable for extended periods of time (e.g., during the determination of their hydrodynamic and equilibrium binding parameters). In previous work with unoccupied and occupied Type I and Type II receptors for adrenal steroids in brain cytosol we have systematically examined the stabilizing effectiveness of a number of factors including temperature, ionic strength, chelators, molybdate, dithiothreitol, dextran-coated charcoal adsorption, etc. (Gray, 1982; Densmore et al., 1984a; Densmore et al., 1984b; Densmore and Luttge, 1985; Densmore et al., 1986a; Densmore et al., 1986b; Emadian and Luttge, 1986; Emadian et al., 1986); however, even with our best buffer

formulations, we still observe a time- and temperature-dependent loss of unoccupied Type I receptor binding capacity (Emadian et al., 1986). Work in other laboratories has shown that polyhydric compounds (e.g., sugars and polyvalent alcohols) can stabilize a wide range of globular proteins, including a number of enzymes and receptors, against time-dependent thermal degradation (Feil et al., 1972; Rafestin-Oblin et al., 1977; Back et al., 1979; Ogle, 1980; Gekko and Timasheff, 1981a & b; Ogle, 1983). In view of these successes, the experiments presented here examine the effects of a number of mono- and polyhydric compounds on the stability and binding capacity of unoccupied Type I receptors for [^{3}H]ALDO in brain cytosol. The results from these studies suggest that spontaneous conformational changes that occur as a consequence of interaction between these receptors and their aqueous cytosolic environment may ultimately lead to the infolding of hydrophobic steroid binding site(s) on the surface of Type I receptors.

Materials and Methods

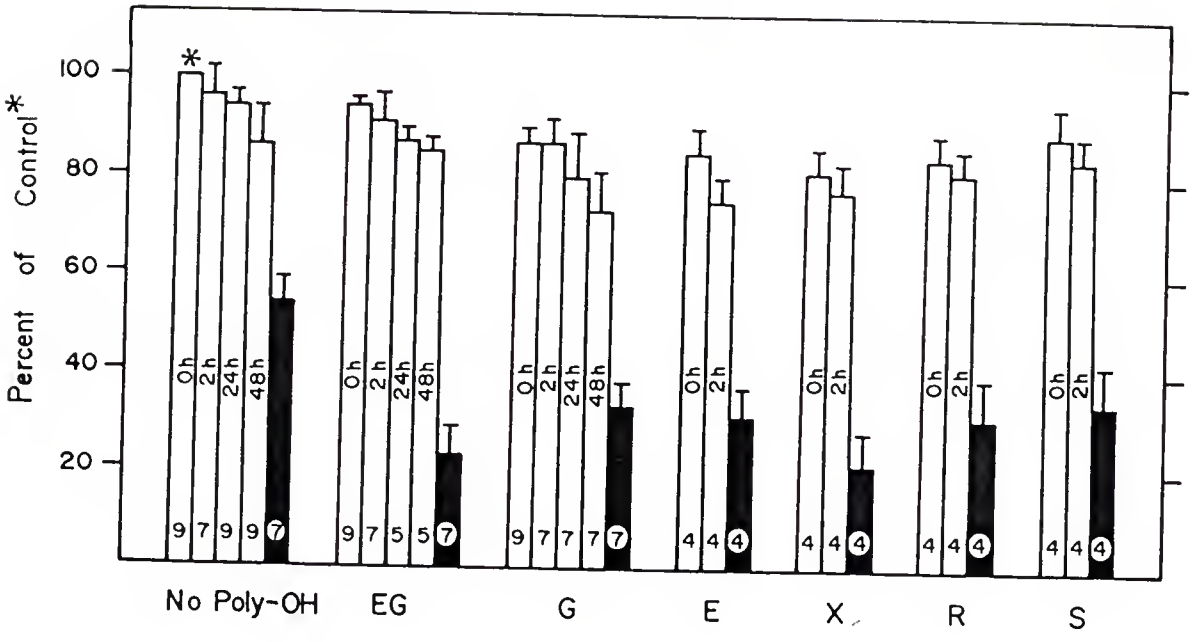
Chemicals. Reagent grade poly- and monohydric compounds were purchased from Sigma.

Buffers. The final concentration of mono- and polyhydric compounds in cytosol was obtained by adding concentrated stock buffers containing each compound to the cytosol to yield the desired concentration of that compound. The pH is all buffers was adjusted to 7.60 at 0-2°C. All other materials and methods were as described in Chapter II.

Results

Fig. 3-1 shows that a 10% (weight:volume) final concentration of di- (ethylene glycol, EG), tri- (glycerol, G), tetra- (erythritol, E), penta- (xylitol, X and ribitol, R) or hexavalent (sorbitol, S) alcohols

Figure 3-1. Effects of Polyhydric Compounds on the Stability of Unoccupied Type I Receptors in Whole Brain Cytosol. Cytosol prepared in Buffer A was either diluted with additional Buffer A (No Poly-OH) or adjusted to 10% (weight:volume) ethylene glycol (EG), glycerol (G), erythritol (E), Xylitol (X), ribitol (R) and sorbitol (S) using a concentrated HEPES-buffered solution of each compound. Subsequently, the cytosol was divided into two parts: one part was incubated immediately with 10 nM [^{3}H]ALDO plus 5 uM RU 26988 and either with (B_{NS}) or without (B_{T}) 2 uM [^{1}H]ALDO for 24 h 0°C (0-h open bars). The second part was aged either from 2 to 48 h at 0°C (2-, 24- and 48-h open bars) or for 2 h at 22°C (closed bars) prior to incubation with the steroids. At the end of the incubation period, macromolecular-bound from free steroids were separated on Sephadex G-25 columns and specific binding was determined as described in Chapter II. The number of independent replicate experiments is indicated near the base of each bar. All data presented are based on the percent of the non-aged, "No Poly-OH" control (*) group $\pm$ SEM. 100% = 28 fmole/mg protein.


Percent of Control*
100
80
60
40
20
*
0h
2h
24h
48h
9 7 9 9 7
9 7 5 5 7
9 7 7 7 7
4 4 4
4 4 4
4 4 4
4 4 4
No Poly-OH
EG
G
E
X
R
S

to unlabeled brain cytosol produced a small, but significant ($p < 0.05$), reduction in the binding of [^{3}H]ALDO to Type I receptors for adrenal steroids. Aging unlabeled cytosol at 0°C in the presence of any of these polyhydric compounds did not attenuate, or exacerbate, the loss in Type I receptor binding capacity seen in the control cytosol (No Poly-OH). However, when cytosol was aged for 2 h at 22°C, the reduction in the subsequent binding of [^{3}H]ALDO to Type I receptors in the presence of polyhydric compounds was nearly twice as great as in their absence. Since a 10% concentration of glycerol has also been routinely included by many investigators in buffers used for Type II receptors, we examined the effects this compound on [^{3}H]DEX binding. As shown in Fig. 3-2, the addition of 10% glycerol to brain cytosol prior to incubation with [^{3}H]DEX fails to protect the time- and temperature-dependent loss in Type II receptor binding capacity. It is noteworthy that, in contrast to the results obtained with Type I receptors (Fig. 3-1), glycerol does not exacerbate the loss in binding capacity when unoccupied Type II receptors are aged for 2 h at 22°C.

Because polyhydric compounds failed to stabilize Type I (or Type II) receptor binding capacity, we next investigated the effects of monohydric alcohols on the binding of [^{3}H]ALDO to Type I receptors (Fig. 3-3). In contrast to the data with polyhydric compounds, when a 10% (weight:volume) final concentration of methanol, and especially ethanol, but not propanol, was added to cytosol immediately before the steroids, we observed a significant increase in [^{3}H]ALDO binding to Type I receptors. If cytosol was aged for 2 h at 0°C prior to the addition of steroids, ethanol still produced an increase in the subsequent binding of [^{3}H]ALDO to Type I receptors, whereas methanol

Figure 3-2. Effects of 10% Glycerol on the Stability of Unoccupied Type II Receptors in Whole Brain Cytosol. Cytosol prepared in Buffer D was either diluted with additional Buffer D (No Poly-OH) or adjusted to 10% (weight:volume) glycerol using a concentrated HEPES-buffered solution of glycerol containing 2 mM DTT (G). Subsequently, the cytosol was divided into two parts: one part was incubated immediately with 20 nM [^{3}H]DEX and either with (B_{NS}) or without (B_T) 4 uM [^{1}H]DEX for 24 h at 0°C (0-h open bars). The second part was aged either from 2 to 48 h at 0°C (2-, 24- and 48-h open bars) or for 2 h at 22°C (closed bars) prior to incubation with the ligand. At the end of the incubation period, macromolecular-bound from free steroid was separated on Sephadex G-25 columns and specific binding was determined as described in Chapter II. The results are the mean from 2 independent replicate experiments. All data presented are based on the percent of the non-aged, "No Poly-OH" control (*) group. 100% = 300 fmole/mg protein.

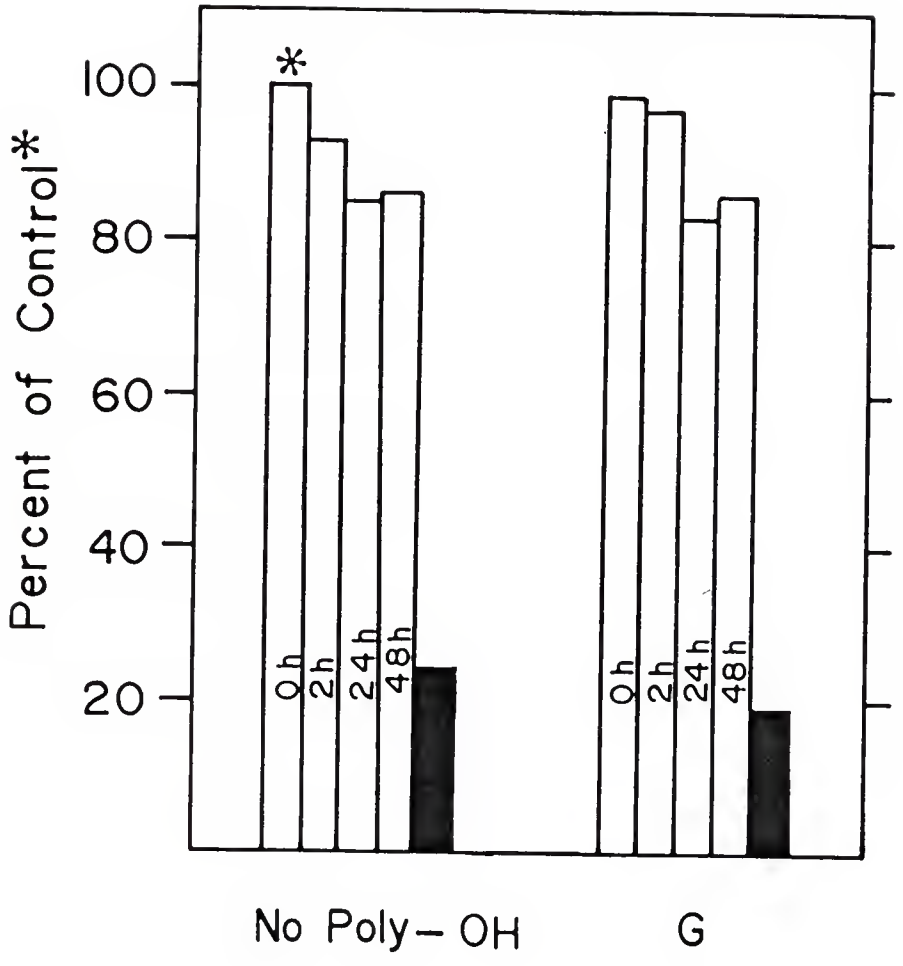
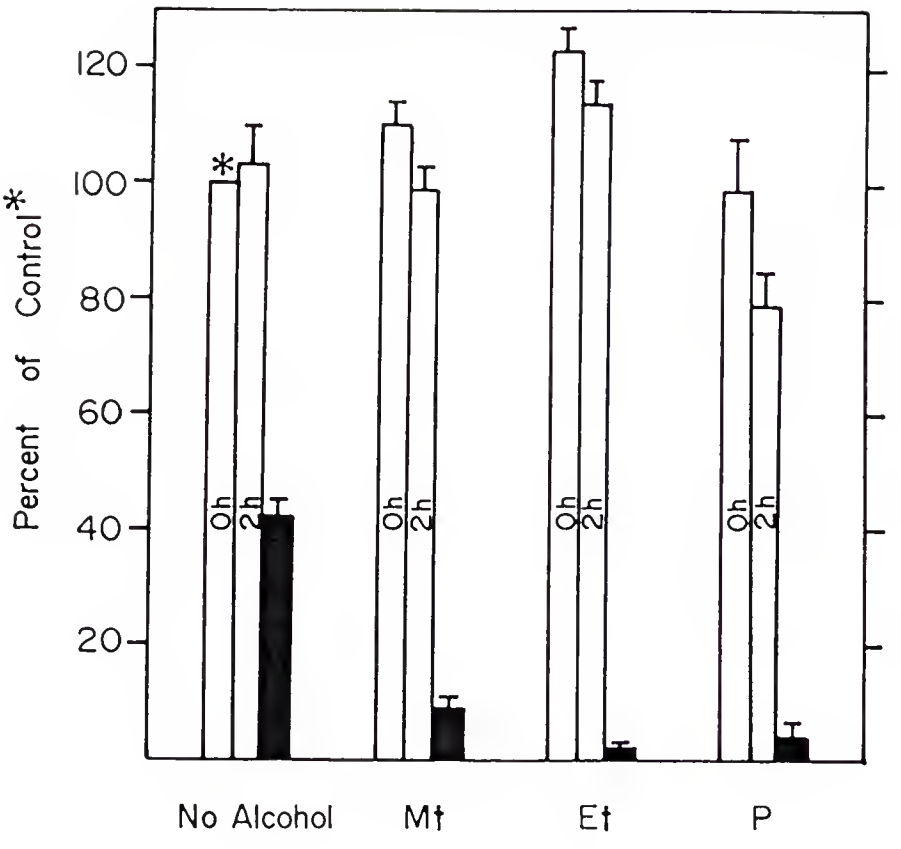

Percent of Control*
100
80
60
40
20
*
0h
2h
24h
48h
0h
2h
24h
48h
No Poly-OH
G

Figure 3-3. Effects of Monohydric Compounds on the Stability of Unoccupied Type I Receptors in Whole Brain Cytosol. Cytosol prepared in Buffer A was either diluted with additional Buffer A (No Alcohol) or adjusted to 10% (weight:volume) methanol (Mt), ethanol (Et) and propanol (P) using a concentrated HEPES-buffered solution of each compound. Subsequently, the cytosol was divided into two parts: one part incubated immediately with 10 nM [^{3}H]ALDO plus 5 uM RU 26988 and either with (B_{NS}) or without (B_{T}) 2 uM [^{1}H]ALDO for 24 h at 0°C (0-h open bars). The second part was aged for 2 h at 0 or 22°C (2-h open and closed bars, respectively) prior to incubation with the steroids. At the end of the incubation period, macromolecular-bound from free steroids were separated on Sephadex G-25 columns and specific binding was determined as described in Chapter II. All data presented are based on the percent of the non-aged, "No Alcohol" control (*) group expressed as the mean $\pm$ SEM from 6 independent replicate experiments. 100% = 18 fmole/mg protein.


Percent of Control*
120
100
80
60
40
20
*
0h
2h
No Alcohol
Mt
Et
P

had no effect and propanol produced a clear reduction in receptor binding capacity. When cytosol preparations were aged for 2 h at 22°C, all three alcohols greatly increased the loss in Type I receptor binding capacity.

An investigation of the effects of various concentrations of ethanol on the binding of [^{3}H]ALDO to cytosolic Type I receptors revealed an inverse U-shaped curve with 10% ethanol producing the greatest binding (120% of control) and 40% ethanol leading to a complete inactivation of Type I receptors (1% of control) (Fig. 3-4). Furthermore, a Scatchard (1949) analysis of the equilibrium binding data revealed that the 10% concentration produced a 46% increase in the K_d and a 23% increase in the B_{max} of [^{3}H]ALDO binding to Type I receptors in brain cytosol (Fig. 3-5).

Discussion

Proposed Mechanism of Action of Poly- and Monohydric Compounds.

For proteins in an aqueous environment, the non-covalent weak intramolecular interactions (i.e., electrostatic forces, hydrogen bonding and Van der Waals forces), as well as the interactions between protein molecules and their surrounding solvent (i.e., hydrophobic interactions), act in concert to stabilize the functionally important tertiary structure of proteins. In some proteins, additional stability may result from cross-linking, metal complex formation and binding to other ions and cofactors; however, it is often held that the hydrophobic interactions, a consequence of net unfavorable interactions between solvent (i.e., water molecules) and non-polar residues in the protein, play the most important role in stabilizing proteins (e.g., Creighton, 1984; Fersht, 1985). Therefore, when proteins are extracted from the

Figure 3-4. Dose-Response Analysis of the Effects of Ethanol on the Binding of ALDO to Type I Receptors in Whole Brain Cytosol. Cytosol prepared in Buffer A was either diluted with additional Buffer A (control, *) or adjusted to 1 to 40% (weight:volume) ethanol using a concentrated HEPES-buffered solution of ethanol. Subsequently, all groups were incubated with 10 nM [^{3}H]ALDO plus 5 uM RU 26988 and either with (B_{NS}) or without (B_{T}) 2 uM [^{1}H]ALDO for 24 h at 0°C. At the end of the incubation period, macromolecular-bound from free steroids were separated on Sephadex G-25 columns and specific binding was determined as described in Chapter II. All data presented are based on the percent of 0% ethanol control (*) group expressed as the mean $\pm$ SEM from 3-12 independent replicate experiments. 100% = 23 fmole/mg protein.

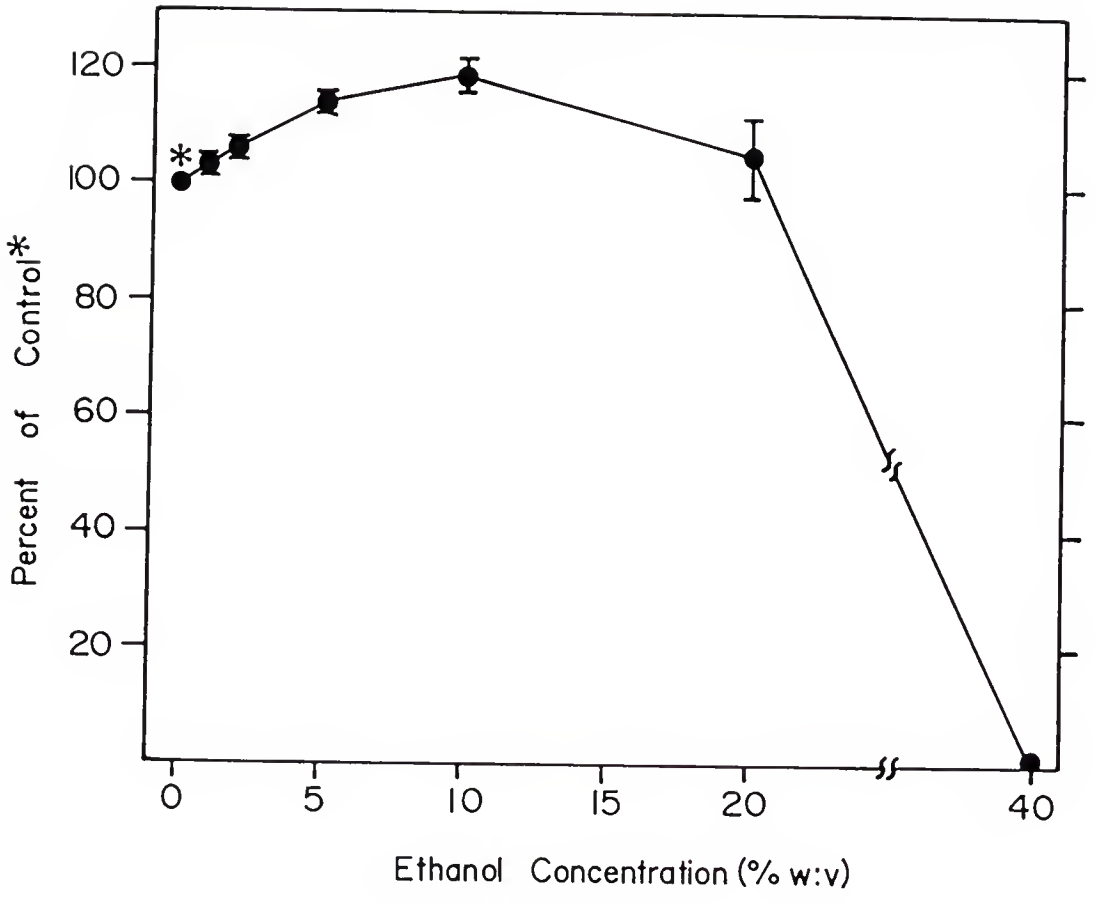
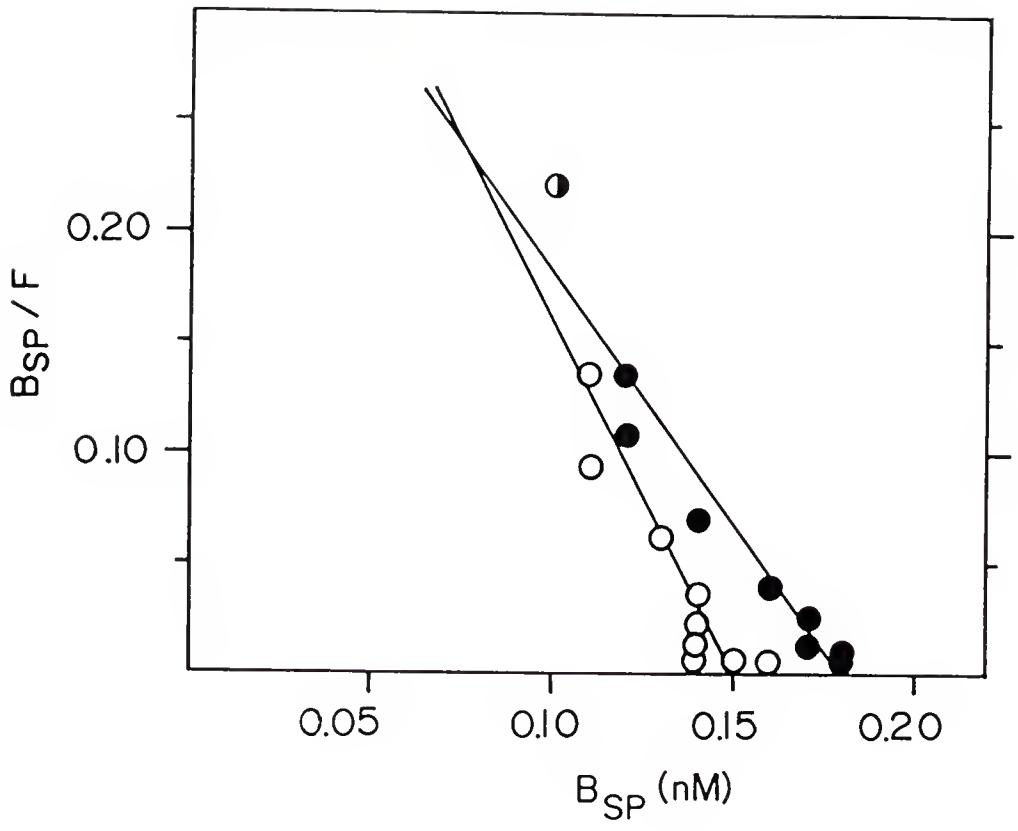

120
100
80
60
40
20
*
Percent of Control*
0
5
10
15
20
40
Ethanol Concentration (% w:v)

Figure 3-5. Scatchard Analysis of the Effects of 10% Ethanol on the Equilibrium Binding Parameters of ALDO Binding to Type I Receptors in Whole Brain Cytosol. Cytosol prepared in Buffer A was either diluted with additional Buffer A (open circles) or adjusted to 10% (weight:volume) ethanol using a concentrated HEPES-buffered solution of ethanol (closed circles). Subsequently, 0.25 ml aliquots from each group was incubated with 0.5 to 40 nM [^{3}H]ALDO plus 500-fold molar excess of RU 26988 and either with (B_{NS}) or without (B_{T}) 200-fold molar excess of [^{1}H]ALDO for 24 h at 0°C. At the end of the incubation period, macromolecular-bound from free steroids were separated on Sephadex G-25 columns and specific binding was determined as described in Chapter II. The Scatchard plot shown is representative from 4 independent replicate experiments. Binding parameters were calculated using least square linear regression. Open circles: B_{max} = 21 fmole/mg protein, $K_{d} = 3.0 \times 10^{-10}$ M. Closed circles: B_{max} = 26 fmole/mg protein, $K_{d} = 4.4 \times 10^{-10}$ M.


0.20
0.10
B_{SP} / F
0.05
0.10
0.15
0.20
B_{SP} (nM)

intracellular environment into a medium that is more aqueous than cytoplasm, it is not uncommon for them to undergo spontaneous conformational changes leading to their functional inactivation. To protect proteins against such in vitro denaturation, various strategies have been utilized to reduce unfavorable solvent-solute interactions.

In a series of studies by Gerlsma (1968 & 1970) and Gerlsma and Stuur (1972 & 1974), polyhydric compounds were shown to increase the melting temperature (T_m) of chymotrypsinogen A, ribonuclease and lysozyme and hence stabilize the native conformation of these proteins at a higher temperature. In contrast, monohydric alcohols were found to destabilize these proteins (i.e., lower the T_m). On a molecule per molecule basis, the increase in the T_m produced by polyhydric compounds was shown to be related directly to the number of hydroxyl groups per molecule of the polyhydric compound, whereas the extent of destabilization produced by monohydric compounds were shown to depend on the length of their alkyl chain. On the basis of these results, Gerlsma and Stuur (1972 & 1974) proposed that polyhydric compounds stabilize proteins by decreasing the hydrogen bond rupturing capacity of the solvent, whereas monohydric compounds augment such solvent-induced hydrogen bond rupturing capacity. This proposed mechanism is somewhat different from that set forth earlier by McDuffie et al. (1962), who maintained that polyhydric compounds stabilize proteins by inducing the tetrahedral conformation of water molecules at higher temperatures which, in turn, increase the hydrophobic interactions. In support of this hypothesis, Oakenfull & Fenwick (1979) showed that hydrophobic

interactions in aqueous-organic environments are maximized when the three-dimensional hydrogen-bonded structure of water is most developed.

In a recent quantitative analysis by scanning calorimetric technique, Back et al. (1979) have shown that sucrose and glycerol strengthen pairwise hydrophobic interactions in aqueous solutions of ovalbumin, lysozyme, α-chymotrypsinogen and conalbumin; however, both of these polyhydric compounds were also shown to reduce the driving force for transfer of a hydrophobic group from an aqueous to a nonpolar environment. Other workers have argued that glycerol, the most common polyhydric compound used to stabilize the activity and native structure of enzymes and proteins (Feil et al., 1972; Rafestin-Oblin et al., 1977; Ogle, 1980; Dias et al., 1981; Korge and Timpmann, 1983; Livesey et al., 1983; Ogle, 1983; Ramaley and Vasantha, 1983; Fojo et al., 1985), increases the hydrophobic interactions in chymotrypsinogen A, α-chymotrypsin, γ-lactoglobulin and ribonuclease A by being preferentially excluded from the immediate domain of these proteins (Gekko and Timasheff, 1981a & b; Timasheff et al., 1976). Collectively, these results suggest that polyhydric compounds tend to strengthen the pairwise hydrophobic interactions in proteins by promoting the three-dimensional hydrogen-bonded structure of water, hence stabilizing the native, more folded conformation of proteins.

Possible Mode of Action of Poly- and Monohydric Compounds on Unoccupied Type I receptors in Crude Cytosol.

Recently we reported that, in contrast to unoccupied Type II adrenal steroid receptors, unoccupied Type I adrenal steroid receptors lose their binding capacity for [^{3}H]ALDO in crude brain cytosol in a time- and temperature-dependent manner even in the presence of DTT, molybdate and/or glycerol (Emadian et al., 1986). However, as discussed

above, since protein molecules are thought to exist in a dynamic state in aqueous environments (Creighton, 1984), we now think that our earlier findings with Type I receptors could be a reflection of the transient fluctuations in the three-dimensional structure of the unoccupied receptors in cytosol resulting in an exclusion of [^{3}H]ALDO from its specific hydrophobic binding site.

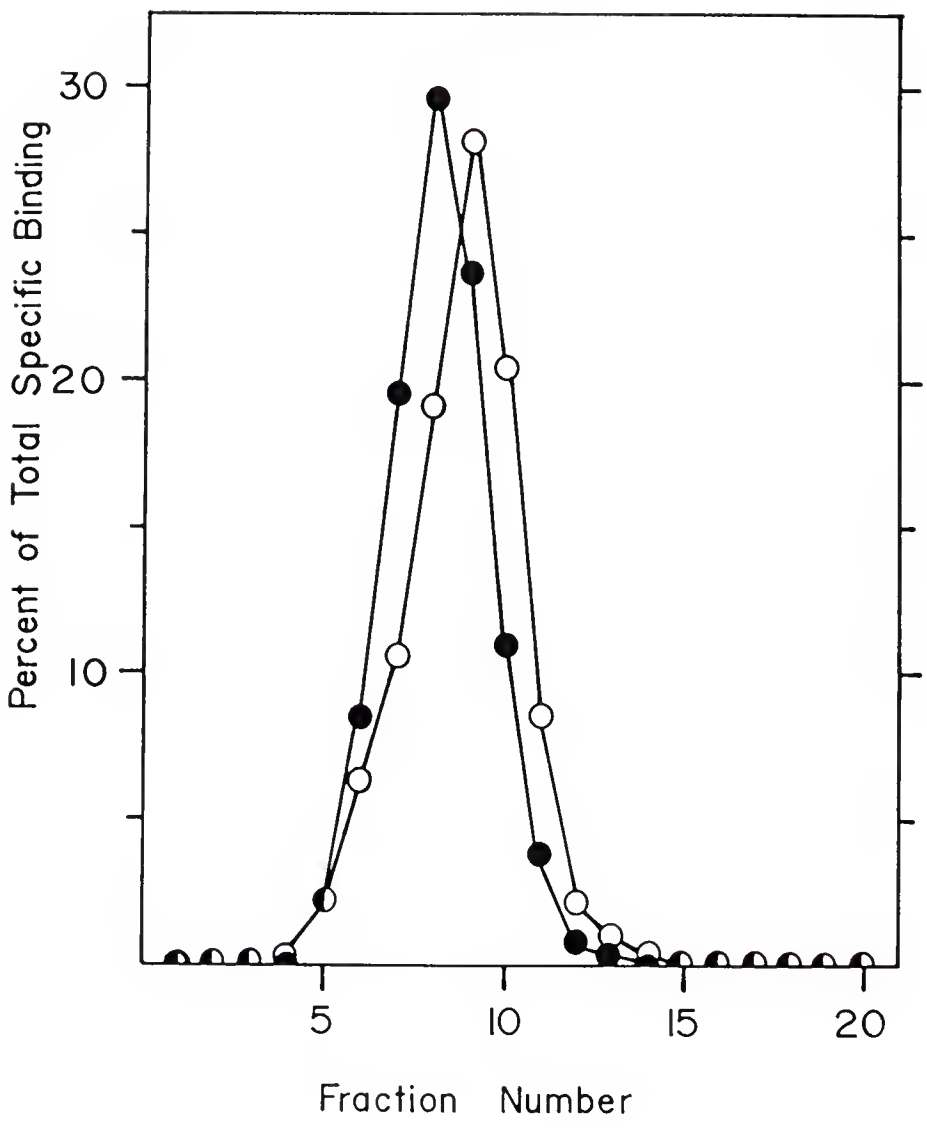
To arrive at this hypothesis, we first investigated the effects polyhydric compounds with increasing ability to promote the tetrahedral conformation of water molecules (i.e., di-, tri-, tetra-, penta-, and hexavalent alcohols), hence an increased ability to enhance intramolecular hydrophobic interactions, on the [^{3}H]ALDO binding capacity of unoccupied Type I receptors. As shown in Figure 3-1, all of the polyhydric compounds tested failed to stabilize unoccupied Type I receptors, in fact, all of them produced a slight reduction in the specific binding of [^{3}H]ALDO, especially when cytosol was aged for 2 h at 22°C prior to binding determinations. These results suggest that increases in the intramolecular hydrophobic interactions within the unoccupied receptor, following the addition of polyhydric compounds to aqueous cytosol preparations, may result in the preferential exclusion of the steroid from its hydrophobic binding sites. If so, one would expect that a reduction in intramolecular hydrophobic interactions should increase the binding of [^{3}H]ALDO to Type I receptors. It is noteworthy that, compared to Type I receptors, inclusion of 10% glycerol in our buffers did not affect the binding of ligand to Type II receptors (Figure 3-2). The differential effects of glycerol on Type I and Type II receptors strongly suggested that there might be differences in the overall surface hydrophobicity of these receptors. In fact, a

comparison of the surface hydrophobic properties of these receptors by glass fiber filter assay (Luttge et al., 1984) and hydrophobic interaction chromatography (Figure 3-6) revealed a greater overall surface hydrophobicity for Type I receptors.

To test the hypothesis that a reduction in hydrophobic interactions may increase the binding of ligand to Type I receptors, we next investigated the effects of adding monohydric alcohols to brain cytosol. As predicted, when compared to the non-aged, "no-alcohol" group, a 10% final concentration of ethanol in cytosol increased the binding of [^{3}H]ALDO to Type I receptors by about 20% (Figs. 3-3 and 3-4). This effect was reflected in Scatchard plots by an increase in the B_{max} (Fig. 3-5). In spite of this increase, the ethanol-induced reductions in intramolecular hydrophobic interactions also appear to reduce the stability of unoccupied Type I receptors when they are aged at 0°C, and especially at 22°C. This increase in the lability of the unoccupied receptor could easily account for the K_d shift seen in the Scatchard plot (Fig. 3-5) (see Beck and Goren, 1983 for a theoretical explanation of this phenomenon) and may be similar to the previously reported temperature-dependent effects of ethanol on the stability of ribonuclease and glutamate dehydrogenase (Shukuya and Schwert, 1960; Brandts and Hunt, 1967).

In summary, the results described in this chapter provide indirect support for the hypothesis that [^{3}H]ALDO binding to Type I receptors is dependent upon the exposure and stabilization of hydrophobic steroid binding sites (and possibly other hydrophobic residues) on the surface of the receptors. Since the comparatively stable <u>in vivo</u> conformation of Type I receptors presumably allows for optimal ligand

Figure 3-6. Comparison of the Surface Hydrophobic Properties of ALDO-Type I and DEX-Type II Receptor Complexes on Pentyl-Agarose Columns. Cytosol prepared in buffer DM was incubated with either 10 nM [^{3}H]ALDO plus 5 uM RU 26988 (to label Type I receptors, open circles) or 20 nM [^{3}H]DEX (to label Type II receptors, closed circles) at 0°C for 48 h for determination of B_T. The determinations of B_{NS} for Type I and Type II receptors were performed in the presence of 2 uM [^{1}H]ALDO and 4 uM [^{1}H]DEX, respectively. Following separation of macromolecular-bound from free steroids on Sephadex G-25 columns, hydrophobic interaction column chromatography was performed as described in Chapter II. The data presented are expressed as a percent of total B_{SP} eluted from pentyl-Agarose columns and are representative from 2 independent replicate experiments.


Percent of Total Specific Binding
30
20
10
5
10
15
20
Fraction Number

binding, it seems likely that these receptors undergo a spontaneous solvent-induced folding upon extraction into the more aqueous cytosolic environment which results in a reduction in binding capacity and stability. Although, the mechanism(s) and/or intracellular factors producing the marked stability of Type I and other steroid receptors *in situ* awaits further experiments, the present findings may provide some clues as to the nature of their properties.

CHAPTER IV
A NOVEL EFFECT OF MOLYBDATE ON THE BINDING OF [^{3}H]ALDOSTERONE TO GEL FILTERED TYPE I RECEPTORS IN BRAIN CYTOSOL

Summary

Recently, we reported that the addition of sodium molybdate to crude cytosol at 0°C results in a dose-dependent reduction in the binding of [^{3}H]ALDO to Type I receptors for adrenal steroids (Emadian et al., 1986). In the experiments outlined here, we show that Sephadex G-25 gel filtration of whole brain cytosol from adrenalectomized and ovariectomized CD-1 female mice in the presence of 2 mM molybdate results in a 30-50% increase in the specific binding of 10 nM [^{3}H]ALDO to Type I receptors above the level of [^{3}H]ALDO binding seen in crude cytosol prepared in 20 mM HEPES buffer (control group). All specific binding determinations were performed in the presence of 5 uM RU 26988 -- a specific synthetic glucocorticoid known to block [^{3}H]ALDO binding to Type II (glucocorticoid) receptors. In Scatchard plots, this molybdate- and gel filtration-induced increase in specific [^{3}H]ALDO binding was reflected as a 37% increase in maximal binding (B_{max}) with no change in the equilibrium dissociation constant (K_d). In contrast, when gel filtration was performed in the absence of molybdate, there was a marked reduction in the subsequent specific binding of [^{3}H]ALDO to Type I receptors. In Scatchard plots, this latter effect was reflected as a 62% reduction in the B_{max} and a 2-fold increase in the K_d when compared to the control group. The addition of 2 mM molybdate immediately following gel

filtration yielded specific [^{3}H]ALDO binding comparable to the control group. Although the addition of 2 mM molybdate prevents the loss in the binding capacity of unoccupied Type I receptors in crude cytosol incubated at 22°C, Type I receptors in gel filtered cytosol were very unstable at 22°C, and appeared insensitive to the protective effects of 2, but not 10-100 mM, molybdate. Two mM dithiothreitol (DTT, a potent sulfhydryl reducing reagent) was unable to prevent the gel filtration-induced loss in [^{3}H]ALDO binding. Moreover, in gel filtration experiments, the data obtained in the presence of 2 mM DTT plus 2 mM molybdate did not differ from those obtained in the presence of 2 mM molybdate alone. The effect of molybdate on Type I receptors caused by its presence during gel filtration of cytosol is clearly different from that seen with these receptors in crude cytosol preparations, as well as those reported in the literature for other steroid receptors. Possible mechanisms of action of molybdate on unoccupied Type I receptors in crude and gel filtered cytosol are discussed.

Introduction

In a series of reports from Pratt's laboratory it was shown that the addition of sodium molybdate to crude cytosol preparations prevents the heat-induced loss in the binding capacity of unoccupied glucocorticoid (Type II) receptors (Nielsen et al., 1977b & c). Subsequently, Toft and Nishigori (1979) showed that this transition element oxyanion reversibly blocks the temperature-dependent transformation (activation) of progesterone receptors to a DNA-binding state. Later, numerous reports have shown similar stabilizing effects of molybdate on other steroid receptors (Nishigori and Toft, 1980;

Shyamala and Leonard, 1980; Miller et al., 1981) and proteins (Defay et al., 1984; Nakada et al., 1985; Denison et al., 1986). In contrast to these findings, recently we have reported that although molybdate prevents the heat-induced inactivation of Type I receptors, at 0°C, this compound leads to a dose-dependent reduction in the subsequent binding of [^{3}H]ALDO in crude whole brain cytosol preparations (Emadian et al., 1986). In the experiments below, we will describe yet another novel effect of molybdate on Type I receptors in Sephadex G-25 gel filtered whole brain cytosol.

Materials and Methods

Chemicals. All chemicals used in the experiments in this chapter were reagent grade quality.

Buffers. The cytosolic concentration of molybdate and DTT were obtained by a 9:1 dilution of cytosol with stock buffers containing a 10-fold concentration of the desired component(s). Under such circumstances, the control group was diluted accordingly with an equivalent volume of Buffer A. The final pH for all buffers was adjusted to 7.60 at 0-2°C.

Sephadex G-25 Gel Filtration. Gel filtered receptor preparations were obtained by loading 0.5 ml aliquots of unlabeled crude cytosol on Sephadex G-25 columns (0.6 x 14.0 cm) pre-equilibrated in appropriate buffer(s) at 4-5°C. The sample on each column was allowed to penetrate the gel and the column was then washed with 1.0 ml buffer. Subsequently, the macromolecular fraction was collected using an additional 1.0 ml homologous buffer.

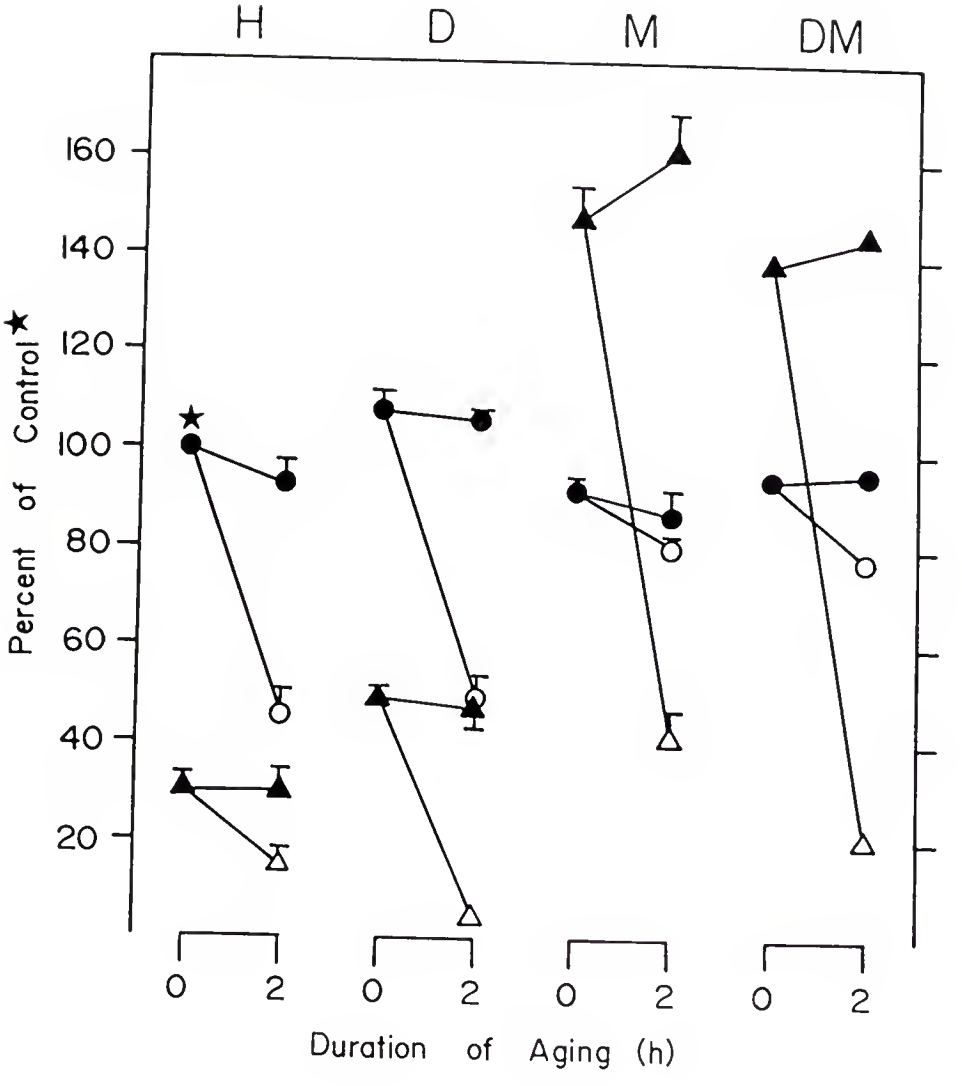
Results

Gel filtration of cytosol prepared in Buffer A through Sephadex G-25 columns (equilibrated and eluted with homologous buffer) led to a 70% reduction in the subsequent specific binding of [^{3}H]ALDO to Type I receptors when compared to the control group (i.e., non-aged crude cytosol prepared in Buffer A) (Fig. 4-1, A). There was no additional loss in the binding capacity of Type I receptors when unlabeled crude or gel filtered cytosol was aged for 2 h at 0°C prior to incubation with steroids; however, when the temperature during aging was raised to 22°C, we observed a 50% reduction in the subsequent Type I receptor binding to [^{3}H]ALDO (Fig. 4-1, A; 2 h aging). With the exception of a slight increase in [^{3}H]ALDO binding at 0°C, similar results were obtained when the above procedures were repeated in the presence of 2 mM DTT (Fig. 4-1, D). This latter increase in B_{SP} values was due to lower B_{NS} values in crude and especially gel filtered cytosol preparations containing 2 mM DTT. It should also be noted that unoccupied Type I receptors in gel filtered cytosol containing DTT appeared to be more susceptible to the 22°C aging-induced loss in ligand binding.

In agreement with our earlier report (Emadian et al., 1986), at 0°C, the addition of 2 mM molybdate to unlabeled crude cytosol produced a slight reduction in the binding of [^{3}H]ALDO to Type I receptors (Fig. 4-1, M). However, when the molybdate-containing cytosol was subjected to Sephadex G-25 gel filtration, we observed a 50% increase in the subsequent binding of [^{3}H]ALDO to Type I receptors. Whereas aging unlabeled crude or gel filtered cytosol in Buffer M at 0°C for 2 h had no significant effect on the measurable Type I receptor binding, aging the unlabeled gel filtered, but not crude, cytosol at 22°C for 2 h

Figure 4-1. Effects of Gel Filtration, Molybdate and/or Dithiothreitol on the Binding Capacity and Stability of Unoccupied Type I Receptors from Whole Brain Cytosol. Nine parts cytosol prepared in Buffer A was diluted with either one part Buffer A (A) or adjusted to 2 mM dithiothreitol (D), 2 mM molybdate (M) or 2 mM dithiothreitol plus 2 mM molybdate (DM) using one part Buffer A containing a 10-fold concentration of the respective component(s). Each group either underwent the gel filtration procedure (for detail see "Materials and Methods" section) at 4-5°C (triangles) or was left undisturbed at 4-5°C (circles) during this process. Subsequently, a portion of the crude or gel filtered cytosol from each group was either incubated with 10 nM [^{3}H]ALDO and 5 uM RU 26988 and minus (B_T) or plus (B_{NS}) 2 uM [^{1}H]ALDO at 0°C for 24 h for the determination of specific binding or was aged at 0 (solid symbols) or 22°C (open symbols) for 2 h prior to incubation with the steroids. Gel filtration reduced the cytosolic protein concentration from 7.3 to 3.3 mg/ml. The data presented are from at least 5 independent replicate experiments (except for the DM group) and are expressed as a percent of control* (i.e., specific binding measured in non-aged crude cytosol prepared in Buffer A) $\pm$ standard error of the mean (SEM).

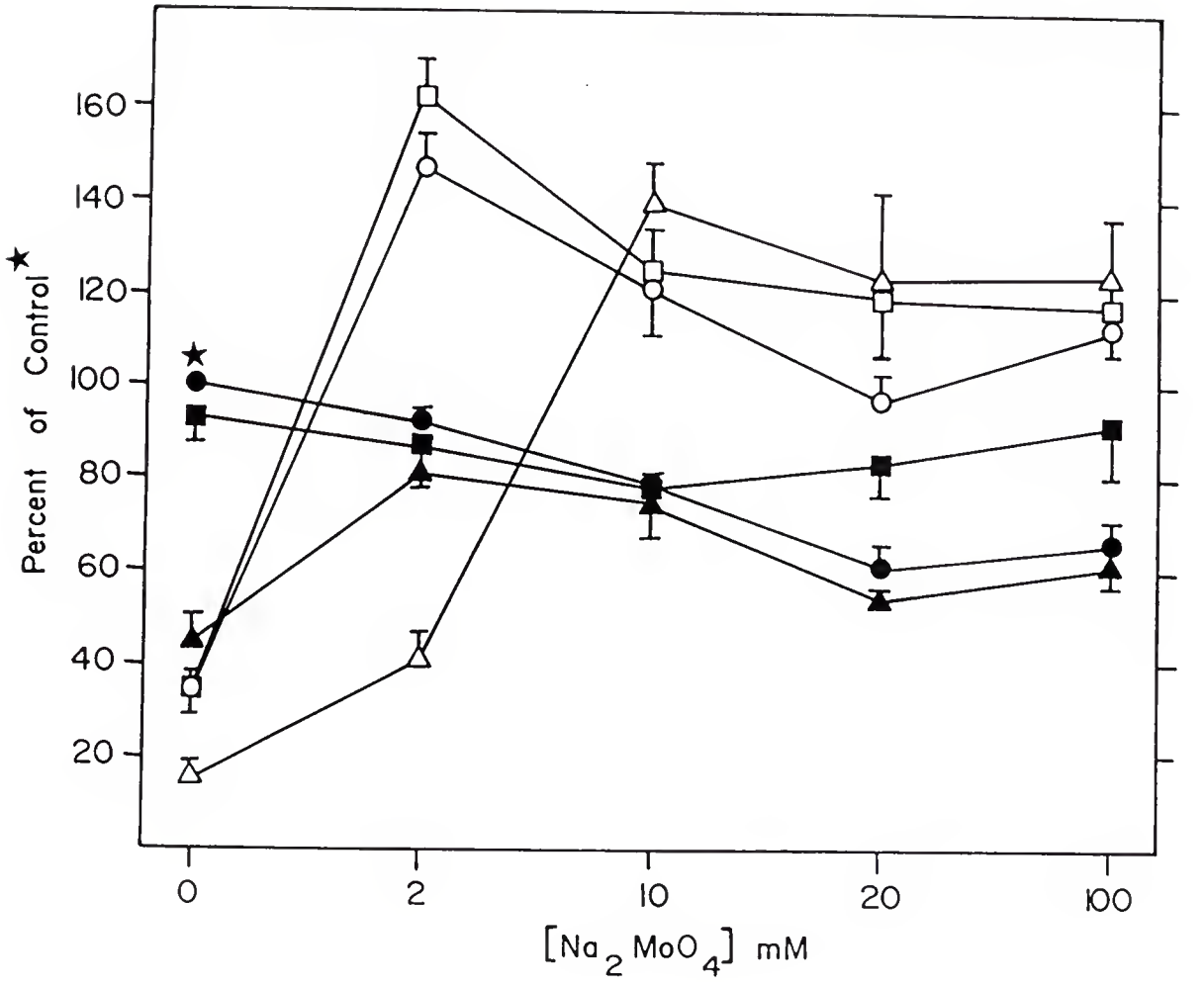
100% = 21 fmole/mg protein.


H
D
M
DM
160
140
120
100
80
60
40
20
Percent of Control★
0
2
0
2
0
2
0
2
Duration of Aging (h)

led to a marked loss in the binding capacity of these receptors (Fig. 4-1, M; 2 h aging). The data obtained in the presence of Buffer DM did not differ appreciably from those described in the presence of Buffer M with the exception, once again, of a slight exacerbation of the heat-induced loss in the binding of [^{3}H]ALDO to Type I receptors in gel filtered cytosol following 2 h aging at 22°C (Fig. 4-1, DM; 2 h aging).

To determine the concentration of molybdate needed to produce the highest increase in the binding of [^{3}H]ALDO to Type I receptors during gel filtration, we conducted a dose-response analysis of the effects of this compound on Type I receptors during the gel filtration procedure. Consistent with our previous observations (Emadian et al., 1986), the addition of molybdate to HEPES buffer led to a dose-dependent loss in [^{3}H]ALDO specific binding to Type I receptors in crude cytosol at 0°C (Fig. 4-2). Aging the crude cytosol for 2 h at 0°C reduced the loss in [^{3}H]ALDO binding seen with the two highest doses of molybdate. In non-aged gel filtered cytosol, the maximal increase in [^{3}H]ALDO binding to Type I receptors occurred at 2 mM molybdate, with 10-100 mM concentrations producing binding levels comparable to those seen in the absence of molybdate (i.e., control group). It is noteworthy that except for when molybdate was absent during gel filtration, at all concentrations of this compound tested, the measurable Type I receptor binding was higher in the non-aged gel filtered cytosol than non-aged crude cytosol containing the same dose of molybdate. Furthermore, at all concentrations of molybdate tested, aging unlabeled gel filtered cytosol for 2 h at 0°C, produced Type I receptor binding comparable to the non-aged gel filtered preparations

Figure 4-2. Dose-Response Analysis of the Effects of Gel Filtration and Molybdate on the Binding Capacity and Stability of Unoccupied Type I Receptors from Whole Brain Cytosol. Nine parts cytosol prepared in Buffer A was diluted with one part Buffer A containing 0, 20, 100, 200 or 1000 mM molybdate to bring the cytosol to a final concentration of 0, 1, 2, 10, 20 or 100 mM molybdate, respectively. Each group then either underwent the gel filtration procedure at 4-5°C (open symbols) or was left undisturbed at 4-5°C during this process (solid symbols). Subsequently, a portion of cytosol from each group was either incubated with 10 nM [^{3}H]ALDO and 5 uM RU 26988 minus (B_T) or plus (B_{NS}) 2 uM [^{1}H]ALDO at 0°C for 24 h for the determination of specific binding (open and solid circles) or was aged at 0 (open and solid squares) or 22°C (open and solid triangles) for 2 h prior to incubation with the steroids. Gel filtration reduced the cytosolic protein concentration from 7.3 to 3.3 mg/ml. The data are from at least 4 independent replicate experiments and are expressed as a percent of control* (i.e., specific binding measured in non-aged crude cytosol prepared in Buffer A) $\pm$ standard error of the mean (SEM). 100% = 18 fmole/mg protein.


160
140
120
100
80
60
40
20
Percent of Control
★
0
2
10
20
100
[Na2MoO4] mM

containing the same doses of molybdate. Consistent with the results in Fig. 4-1, Type I receptors in unlabeled gel filtered cytosol containing 0 (i.e., Buffer A) or 2 mM molybdate (i.e., Buffer M) were found to be extremely labile when aged for 2 h at 22°C; however, increasing the concentration of molybdate to 10-100 mM prevents this loss in binding capacity. Moreover, as reported earlier (Emadian et al., 1986), when crude, non-gel filtered cytosol preparations are aged for 2 h at 22°C, the highest level of [^{3}H]ALDO binding was observed in the presence 2 mM molybdate. Further increases in the concentration of molybdate resulted in lower levels of [^{3}H]ALDO binding, comparable to those measured in non-aged crude cytosol containing the same concentration of molybdate.

To gain further insight about the nature of the effects of gel filtration on the binding of Type I receptors in the presence and absence of molybdate, Scatchard (1949) analyses of the equilibrium binding data were performed (Fig. 4-3, Table 4-1). Gel filtration of cytosol in Buffer M produced no effect on the K_d, but increased the B_{max} of Type I receptors for [^{3}H]ALDO by 36% above the level seen in crude cytosol prepared in Buffer A (control). Gel filtration of cytosol in Buffer A produced a 62% reduction in the B_{max} and a 2-fold increase in the K_d of [^{3}H]ALDO binding to Type I receptors. The presence of 2 mM molybdate in crude cytosol produced a slight increase in the K_d, but had no apparent effect on the B_{max} of Type I receptors when compared to the control group.

To see whether the gel filtration-induced loss in Type I receptor binding capacity observed in Buffer A was reversible, we investigated the effects of 2 mM molybdate added to cytosol at different time

Figure 4-3. Effects of Molybdate and/or Gel Filtration on the Equilibrium Binding Parameters of ALDO Binding to Unoccupied Type I Receptors from Whole Brain Cytosol. Nine parts cytosol prepared in Buffer A was diluted with either one part Buffer A (solid circles and triangles) or one part Buffer A containing 20 mM molybdate to bring the cytosol to a final concentration of 2 mM molybdate (open circles and triangles). Subsequently, each group either underwent the gel filtration procedure (triangles) at 4-5°C or was left undisturbed at 4-5°C during this process (circles). All groups were then incubated at 0°C with 0.1-40 nM [^{3}H]ALDO plus a 500-fold excess of RU 26988 and either with (B_{NS}) or without (B_T) a 200-fold excess of [^{1}H]ALDO at 0°C for 24 h for the determination of specific binding. The concentration of free [^{3}H]ALDO was determined in every tube by subtracting B_T from the total radioactivity present. The values for B_{max} and K_d were obtained from the x-intercept and negative inverse of the slope for each line, respectively, by least square linear regression method. Gel filtration reduced the cytosolic protein concentration from 7.3 to 3.3 mg/ml. The plots are representative of 2 independent replicate experiments.

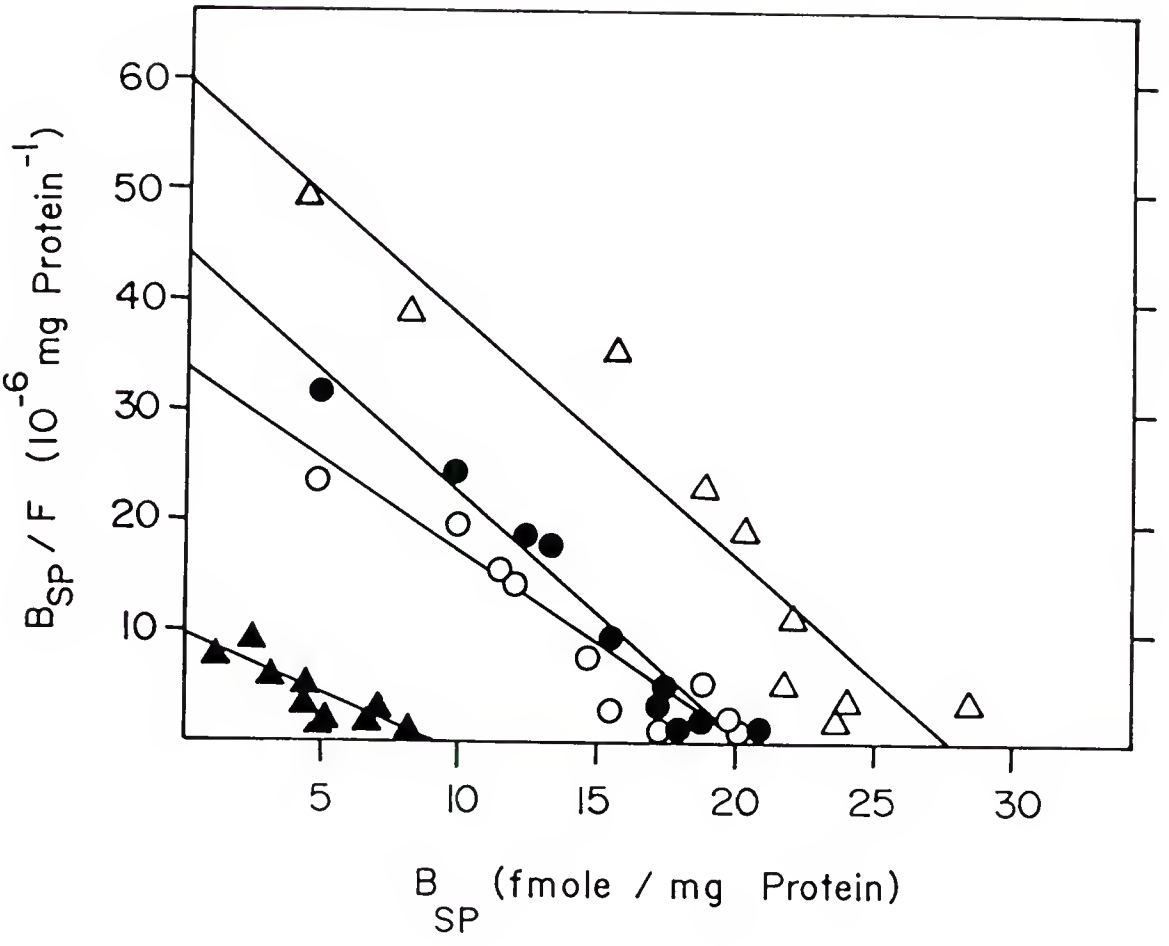

60
50
40
30
20
10
5
10
15
20
25
30
B_{SP} / F (10^{-6} mg Protein^{-1})
B_{SP} (fmole / mg Protein)

Table 4-1

Binding Parameters for Type I Receptors Obtained from Crude and Gel Filtered Cytosol in the Presence and Absence of 2 mM Molybdate[a]

Na_2MoO_4	G-25	K_d[b]	B_{max}[c]	- r
+	+	0.45	27.4	0.95
-	-	0.45	20.1	0.97
+	-	0.60	20.3	0.96
-	+	0.82	7.6	0.89

[a] Experimental conditions are described under Fig. 4-3.

[b] 10^{-9} M.

[c] 10^{-15} mole/mg protein.

intervals following gel filtration. As shown in Fig. 4-4, adding 2 mM molybdate to cytosol immediately following gel filtration in the presence of Buffer A led to a significant recovery of Type I receptor binding capacity; however, the magnitude of this recovery was only sufficient to increase [^{3}H]ALDO binding to the levels seen in the control group and never to the levels observed in cytosol gel filtered in the presence of 2 mM molybdate. The magnitude of this recovery was also time-dependent, since only molybdate added 1 to 5 h following gel filtration led to a significant recovery of the binding capacity of Type I receptors. The addition of molybdate to unlabeled gel filtered cytosol 24 h following gel filtration did not produce any increase in the binding capacity of Type I receptors. The effects of 2 mM molybdate on Type I receptor binding capacity in crude and gel filtered cytosol were comparable to those described in Figs. 4-1 and 4-2.

A number of earlier reports have implicated the role of endogenous small molecular weight "factors" in cytosol as possible modulators of hormone binding to glucocorticoid receptors and subsequent activation (transformation) and DNA binding of the steroid-receptor complexes (Bailly et al., 1977; Ishohashi et al., 1980; Leach et al., 1982; Dahmer et al., 1984). Since during Sephadex G-25 gel filtration many small molecular weight components are removed from cytosol, we sought to investigate the effects of dilution of cytosol (which in effect dilutes the concentration of endogenous factor(s) possibly involved in the inhibition of [^{3}H]ALDO binding to Type I receptors) on the binding capacity of these receptors. As shown in Fig. 5-5, a 2-fold dilution of crude cytosol prepared in Buffer A or incubated with 2 mM molybdate for 2 h prior to dilution, produced Type I receptor binding comparable to

Figure 4-4. Stability of Unoccupied Type I Receptors from Whole Brain Cytosol Following Gel Filtration in the Absence of Molybdate: Effects of Molybdate Addition Following Gel Filtration and Aging.

Nine parts cytosol prepared in Buffer A was diluted with either one part Buffer A (-M) or Buffer A containing 20 mM molybdate to bring the cytosol to a final concentration of 2 mM molybdate (+M, solid bars). Each group then either underwent the gel filtration procedure at 4-5°C (+ G-25) or was left undisturbed at 4-5°C during this process (- G-25). Subsequently, the group that was gel filtered in the absence of molybdate was divided into two parts. One part was brought to 2 mM molybdate immediately or 1, 2, 5 and 24 h following gel filtration (shaded bars). The second part was diluted accordingly with Buffer A immediately or 1, 2, 5 and 24 h (hash marked bars) following gel filtration. All other groups were diluted accordingly with appropriate buffers. Following these steps, all groups incubated with 10 nM [^{3}H]ALDO and 5 uM RU 26988 minus (B_T) or plus (B_{NS}) 2 uM [^{1}H]ALDO at 0°C for 24 h for the determination of specific binding. Gel filtration reduced the cytosolic protein concentration from 7.3 to 3.3 mg/ml. The data are from at least 3 independent experiments and are expressed as a percent of control* (i.e., specific binding measured in non-aged crude cytosol prepared in Buffer A) $\pm$ standard error of the mean (SEM). 100% = 26 fmole/mg protein.

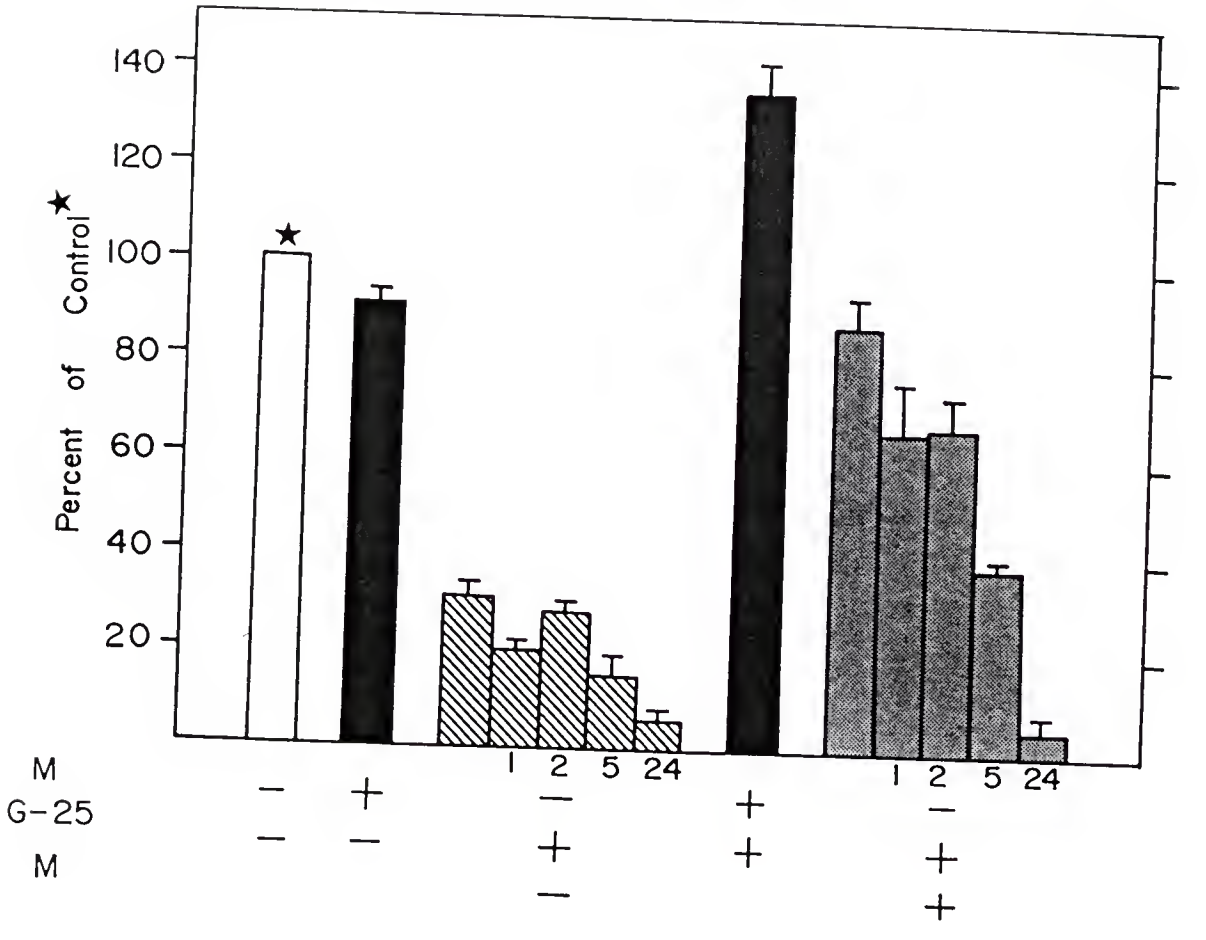
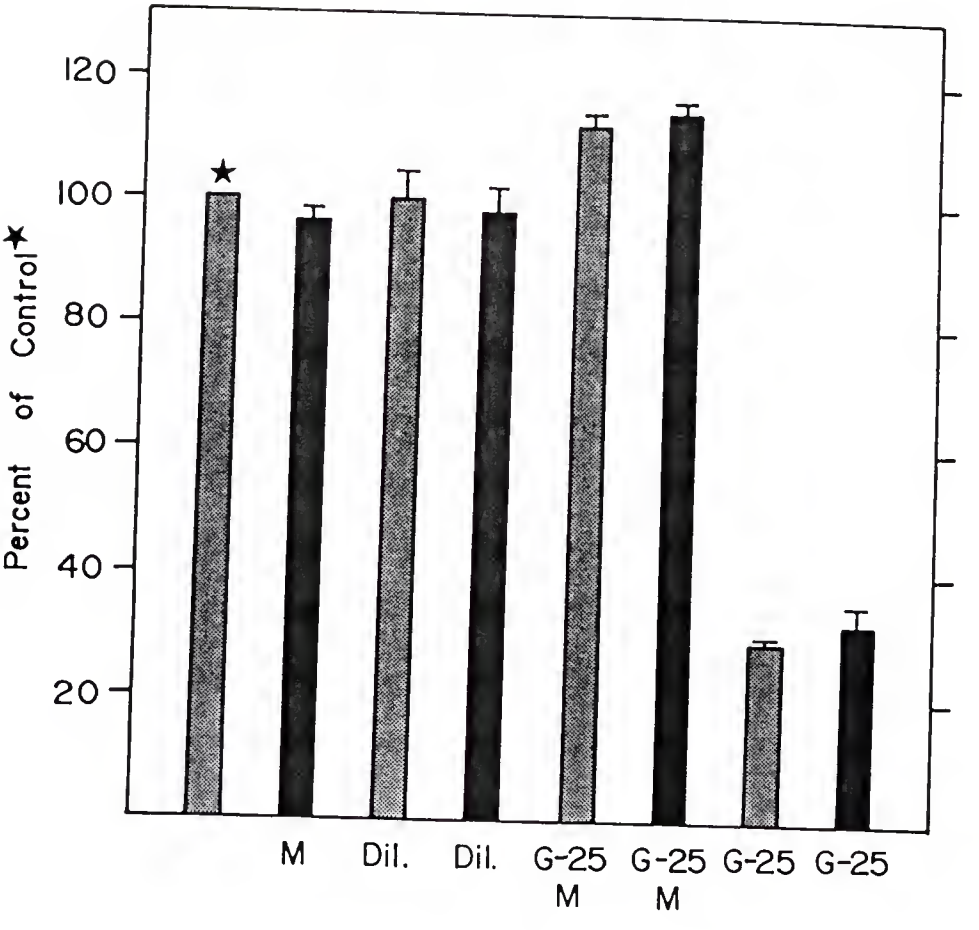

Percent of Control★
140
120
100
80
60
40
20
★
1
2
5
24
1
2
5
24
M
G-25
M
–
–
–
+
–
–
+
–
+
+
+
–
+
+

Figure 4-5. Effects of Dilution and/or Molybdate on the Binding Capacity of Unoccupied Type I Receptors from Whole Brain Cytosol.

Nine parts cytosol prepared in Buffer A was incubated at 0°C for 2 h with either one part Buffer A (shaded bars) or Buffer A containing 20 mM molybdate to bring the cytosol to a final concentration of 2 mM molybdate (solid bars). One group either underwent the gel filtration procedure at 4-5°C in columns pre-equilibrated with (G-25, M) or without (G-25) 2 mM molybdate. A second group was diluted by 2-fold (Dil.) using either Buffer A or Buffer M, where appropriate. A third batch of undiluted crude cytosol in Buffer A (the control*) or Buffer M (M) along with the "Dil." groups remained at 4-5°C during the gel filtration process. Subsequently, all groups were incubated with 10 nM [^{3}H]ALDO and 5 uM RU 26988 minus (B_T) or plus (B_{NS}) 2 uM [^{1}H]ALDO at 0°C for 24 h for the determination of specific binding. Dilution and gel filtration reduced the cytosolic protein concentration from 7.3 to 3.6 and 3.3 mg/ml, respectively. The data are from 4 independent replicate experiments and are expressed as a percent of control* $\pm$ standard error of the mean (SEM). 100% = 25 fmole/mg protein.


120
100
80
60
40
20
Percent of Control★
★
M
Dil.
Dil.
G-25 M
G-25 M
G-25
G-25

the levels seen in the control group (i.e., non-diluted crude cytosol prepared in Buffer A). Gel filtration of cytosol prepared in Buffer A and/or incubated with 2 mM molybdate for 2 h prior to gel filtration through Sephadex G-25 columns pre-equilibrated in Buffer M produced Type I receptor binding 10 and 15% higher than that seen in the control group, respectively. In contrast, gel filtration of the latter groups through columns pre-equilibrated in Buffer A led to a significant loss in the binding capacity of Type I receptors in both of these preparations.

Discussion

The view that molybdate inhibits heat-, salt- and/or ammonium sulfate-induced inactivation of the ligand binding capacity of unoccupied, and the transformation (activation) of occupied, glucocorticoid Type II and other steroid receptors is well accepted (e.g., Leach et al., 1979; Sando et al., 1979a & b; Nishigori and Toft, 1980; Shyamala and Leonard, 1980; McBlain and Shyamala, 1980; Noma et al., 1980; Housley et al., 1982; Murakami et al., 1982; Blanchardie et al., 1984; Luttge and Densmore, 1984; Luttge et al., 1984a, b & d; Weisz et al., 1984; Miller-Diener et al., 1985; Wilson et al., 1986); however, despite the widespread use of this, and other transition metal oxyanions (e.g., vanadate and tungstate), the exact mechanism(s) by which these actions are mediated still remains unclear. The original reports describing the profound stabilizing effects of molybdate on time- and temperature-dependent inactivation of Type II receptors were based on molybdate's purported phosphatase-inhibitor activity (Nielsen et al., 1977b & c; Richards and Swisloki, 1979). The success of these early studies led to the hypothesis that molybdate prevents both unoccupied

receptor inactivation and occupied receptor transformation by inhibiting enzyme-induced dephosphorylation of the receptor (Sando et al., 1979a & b; Barnett et al., 1980; Housley et al., 1982). However, the observations that molybdate does not prevent alkaline phosphatase-induced inactivation of unoccupied Type II receptors (Leach et al., 1983), that other phosphatase inhibitors, including fluoride and glucose-1-phosphate, do not prevent heat-induced transformation of occupied Type II receptors (Nielsen et al., 1977b) and that molybdate can still exert its stabilizing and transformation inhibiting actions even when working with highly-purified (and presumably phosphatase-lacking) receptor preparations (Weisz et al., 1984; Miller-Diener et al., 1985) clearly suggests that phosphatase inhibition is not the principal mechanism of molybdate's actions on steroid hormone receptors. These and other observations have formed the basis for a new hypothesis that molybdate may stabilize Type II, and possibly other steroid, receptors by direct interactions with the receptor macromolecules. In this regard, the formation of complexes between molybdate and sulfhydryl moiety(ies) (possibly near the steroid binding site(s)) has been proposed (Housley et al., 1982; Blanchardie et al., 1984; Dahmer et al., 1984; Potgieter et al., 1986; Wilson et al., 1986).

Recently, we reported that although molybdate was very effective in crude cytosol in preventing the time-dependent, heat-induced loss in Type I receptor binding capacity, this stabilization was accompanied by a molybdate dose-dependent (and time-independent) loss in the ligand-binding capacity of these receptors (Emadian et al., 1986). Tungstate has been reported to produce a similar dose-dependent reduction in the binding capacity of progesterone receptors; however,

this effect was reflected as an increase in the K_d with no effect on the B_{max} (Murakami et al., 1982), whereas in our work with Type I receptors, the reduction in binding was reflected as a decrease in the B_{max} with no effect on the K_d (Emadian et al., 1986). It is noteworthy that the increase in [^{3}H]ALDO binding to Type I receptors following gel filtration of molybdate-containing cytosol shown in this study, was also reflected as an increase in B_{max} with no effect on K_d (Fig. 4-3).

If we assume that certain populations and/or conformations of Type I receptors may have more steroid binding sites per molecule than other populations and/or conformations, then the data from our previous (Emadian et al., 1986) as well as our present studies suggest that molybdate may mediate its facilitatory and inhibitory actions on Type I receptor binding capacity by stabilizing different populations and/or conformations of the receptor which in turn increase or decrease the total number of specific binding sites available under different assay conditions. The lack of an effect on the K_d during both of these situations suggests that all of the binding sites have a similar affinity for [^{3}H]ALDO. Similarly, the lack of curvature in the Scatchard plots during both of these situations suggests that there are no cooperative (positive or negative) interactions between the [^{3}H]ALDO specific binding sites. Assuming, therefore, that the number of steroid binding sites per Type I receptor molecule is not fixed, then the data from the Sephadex G-25 gel filtration experiments suggests that endogenous small molecular weight factors present in crude cytosol may also be able to modulate this number in much the same fashion as seen with molybdate. The concentration of this factor(s) in

crude cytosol (as well as in cytoplasm) appears to be in excess of that needed for maximal binding, since a 50% reduction in the concentration of this factor(s), as well as the receptor and other macromolecules, as would be produced during Sephadex G-25 gel filtration, was shown to have no effect on the number of [^{3}H]ALDO specific binding sites (Fig. 4-5).

The addition of 2 mM molybdate to cytosol prior to the removal of this putative factor(s) (i.e., prior to gel filtration) produces a subtle reduction in [^{3}H]ALDO binding to Type I receptors (Emadian et al., 1986; Figs. 4-1 through 4-5). Note that this effect is clearly different from the increases in the absolute levels of receptor binding capacity seen when either molybdate-containing or molybdate-free cytosol is gel filtered in the presence of 2 mM molybdate (Figs. 4-1 through 4-5). When 2 mM molybdate is added after gel filtration there is a time-dependent loss in its ability to stabilize/increase Type I receptor binding capacity even when the cytosol is maintained at 0°C (Fig. 4-4). Furthermore, although the addition of 2 mM molybdate either prior to, or during gel filtration, produces a 15-50% increase in binding capacity, this unliganded receptor preparation is very unstable when incubated at 22°C (Figs. 4-1 and 4-2). To stabilize unoccupied Type I receptors during 22°C aging of the gel filtered cytosol, molybdate concentrations above 10 mM were found to be required (Fig. 4-2).

On the basis of these results, we speculate that the addition of 2 mM molybdate prior to, or during gel filtration of brain cytosol induces a thermally-labile conformation of the Type I receptor which has a greater number of binding sites per molecule than found for Type I receptors in crude cytosol (molybdate-free or molybdate-containing).

The reduction in binding seen when higher than 2 mM doses of molybdate are used with gel filtered cytosol, may reflect the stabilization of a thermally-resistant receptor conformation with an intermediate number of [^{3}H]ALDO binding sites. Experiments investigating the above possibilities are currently under way in our laboratory.

CHAPTER V
DIFFERENTIAL INACTIVATION OF TYPE II RECEPTORS FOR ADRENAL STEROIDS IN WHOLE BRAIN CYTOSOL: RECOVERY OF INTACT TYPE I RECEPTORS AND DISCOVERY OF A NEW CLASS OF TYPE II RECEPTORS

Summary

Recently we reported that whereas Type II receptors for adrenocorticosteroids in brain cytosol preparations required a sulfhydryl reducing reagent for optimal ligand binding, Type I receptors manifested maximal binding capacity in the absence of reducing reagents (Emadian et al., 1986). Furthermore, removal of endogenous reducing agents from steroid-free cytosol (prepared in the absence of a reducing reagent) by dextran-coated charcoal (DCC) was found to further exacerbate the ligand binding capacity of Type II, but not Type I, receptors (Emadian et al., 1986). To extend the observations reported previously from this laboratory (Densmore et al., 1984a), in the experiments below, we also found unoccupied Type II, but not Type I, receptors lose their ligand binding capacity in the presence of 300 mM KCl, especially when incubated at 0°C. In this chapter, the differences between Type I and Type II receptors were utilized to differentially inactivate the latter, in order to purify and characterize the nuclear acceptor site(s) specific for Type I receptors in future experiments.

Although unoccupied Type I receptors were insensitive to the effects of the reducing reagent, dithiothreitol (DTT), inclusion of 1 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) completely inactivated the ligand binding capacity of these receptors. This DTNB-induced loss in ligand binding could, however, be reversed by adding a 10-fold excess

concentration of DTT to the DTNB-treated cytosol. In contrast, the effects of DTNB on receptors prelabeled with [^{3}H]ALDO in the presence or absence of RU 26988 was found to be minimal. Consistent with our earlier report (Emadian et al., 1986), DCC pretreatment of steroid-free cytosol led to a marked reduction (80 to 90%) in the binding capacity of Type II, but not Type I, receptors. Although this loss in ligand binding capacity could be reversed by adding 2 mM DTT to the DCC-pretreated cytosol, the magnitude of the recovery was dependent on the concentration and/or the duration of DCC pretreatment. The higher the concentration of DCC and/or the longer the duration of the pretreatment, the less the magnitude of the recovery. Aging the DCC pretreated preparations at 0°C caused a progressive loss in the subsequent ligand binding capacity of unoccupied Type II, but not Type I, receptors. Conversely, a loss in the ligand binding capacity of unoccupied Type II, but not Type I, receptors was observed when untreated cytosol preparations were aged at 0°C in the absence of DTT. Use of a molar excess concentration of a number of synthetic ligands for Type I and Type II receptors (e.g., RU 26988, prorenone and RU 26752) in conjunction with [^{3}H]ALDO or [^{3}H]DEX revealed that the remaining 10 to 20% [^{3}H]DEX binding seen following DCC pretreatment of cytosol was mostly to a class of Type II receptors with chemical properties distinct from the classical Type II receptors. Possible physiological and methodological significance of these findings is discussed.

Introduction

Within the past three decades, numerous reports on the properties as well as functional and physiological relevance of steroid hormone receptors have appeared in the literature. Although much has been done

to expand our knowledge regarding the effects of mineralocorticoid hormones on electrolyte balance (see Chapter I), the volume of research on the properties of the receptors through which these effects are mediated has been minimal by comparison (Liao et al., 1983). This is partially due to the fact that in crude cytosol preparations [^{3}H]ALDO and other readily available radiolabeled synthetic ligands for Type I receptors bind to Type II receptors with appreciable affinity (Rousseau et al., 1972; Funder et al., 1973b; Emadian et al., 1986; Emadian and Luttge, unpublished). Recently, however, the synthesis of a number of very specific ligands for Type II receptors (e.g., RU 26988, RU 28362 and RU 38486) has made the elimination of the confounding contribution of [^{3}H]ALDO-binding to Type II receptors in crude preparations possible (Moguilewsky and Raynaud, 1980; Veldhuis et al., 1982b; Gomez-Sanchez and Gomez-Sanchez, 1983; Wrange and Yu, 1983; Moguilewsky and Philibert, 1984; Emadian et al., 1986). Although this competition method for the elimination of Type II receptors may be acceptable under some experimental conditions (e.g., saturation and equilibrium binding analyses, hydrodynamic parameter characterization studies, etc.), at times, the presence of viable Type II receptors may prove undesirable in crude preparations (e.g., during purification procedure using steroid affinity gels, characterization of specific nuclear acceptor sites for activated [^{3}H]ALDO-Type I receptor complexes, development of specific monoclonal antibodies for Type I receptors, etc.).

Recently, we have demonstrated several <u>in vitro</u> chemical differences between Type I and Type II receptors in brain cytosol which may prove important in the <u>in vivo</u> regulation of these receptors.

For example, we showed that although charcoal-extraction of steroid-free cytosol in HEPES buffer lacking dithiothreitol (DTT) causes a dramatic reduction in Type II binding capacity (possibly by removal of endogenous thioredoxin and NADPH), this treatment was found to have no effect on the binding capacity of Type I receptors in the same preparations (Emadian et al., 1986). Furthermore, DTT, while required for the expression of optimal ligand binding capacity of Type II receptors, was found to have no effect on unoccupied Type I receptors in whole brain cytosol. In the experiments described here, we took advantage of the chemical differences between Type I and Type II receptors and attempted to differentially inactivate the Type II receptors in crude whole brain cytosolic preparations. As a result of this work, we report here on the discovery and possible functional and physiological relevance of a new class of glucocorticoid binders, distinct from the classical Type II receptors.

Materials and Methods

Chemicals. 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, shown to be a very specific sulfhydryl oxidizing reagent, Brucklehurst, 1979), Dextran T70 and activated charcoal (Norite A) were obtained from Sigma Chemical Co. All other chemicals used in these experiments were reagent grade quality.

Buffers. The cytosolic concentration of the component(s) in Buffers D, M_{20} and DM_{20} were obtained by a 9:1 dilution of cytosol with stock buffers containing a 10-fold concentration of the desired component(s). Under such circumstances, the control group was diluted accordingly with an equivalent volume of Buffer A. Similarly, the final concentration of DTNB was obtained by adding one volume of 10 mM

buffered solution of this compound to 9 volumes of cytosol to yield 1 mM concentration of this compound in cytosol. Note that to dissolve 10 mM DTNB in our buffers we had to start with buffers in acidic pH ranges (i.e., 4.5-5.5) and then readjust the pH to 7.60 prior to use. All other buffers were also adjusted to final pH 7.60 at 0-2°C prior to use.

DCC Pretreatment of Cytosol. The dextran-coated charcoal (DCC) mixture was prepared overnight by adding a 1:2 ratio of dextran:charcoal to Buffer A. Just prior to use, the desired volume of DCC suspension was centrifuged at 2,230 x g for 10 min, the supernatant discarded and an equivalent volume of cytosol added to the pellet. The DCC was resuspended by gentle vortexing and the mixture incubated at 0°C for the length of time indicated for each specific experiment. During this incubation, the mixture was vortexed gently every 5 min. At the end of the incubation period, the DCC was pelleted by centrifugation at 2,230 x g for 10 min and the resulting supernatant (i.e., DCC-pretreated cytosol) carefully aspirated for subsequent steps. Control groups underwent identical procedural steps in the absence of DCC in parallel with their DCC-pretreated counterparts.

Results and Discussion

Effects of DTNB on Unoccupied Type I Receptors.

As mentioned above, we have recently shown that in crude steroid-free cytosol preparations, unlike Type II receptors, Type I receptors do not require a sulfhydryl reducing reagent for optimal ligand binding (Emadian et al., 1986). Furthermore, we and others have shown that unoccupied Type II receptors can be completely and reversibly inactivated by specific sulfhydryl oxidizing reagents such as DTNB (Bodwell et al., 1984; Densmore, 1986; Emadian and Luttge, unpublished).

Recently, in their investigation of the role of sulfhydryl groups as a potential regulatory mechanism for Type I receptor binding capacity, Tashima et al. (1984) employed a number of sulfhydryl oxidizing reagents to induce inactivation of the swine kidney cytosolic "[^{3}H]ALDO receptors." "Mild" sulfhydryl oxidizing reagents such as DTNB were found to promote a rapid but DTT-reversible inactivation of ALDO binding capacity. Although this study may be accurate in terms of the reported data, the interpretation of the results must take into account the authors' failure to eliminate [^{3}H]ALDO binding to Type II receptors. Therefore, what Tashima et al. (1984) refer to as "aldosterone receptors," is probably a heterogeneous population of Type I and Type II receptors. Note that kidney is a rich source of Type II receptors (Wrange and Yu, 1983; Emadian and Luttge, unpublished; Luttge and Rupp, unpublished), and that ALDO binds to Type II with appreciable affinity (Rousseau et al., 1972; Funder et al., 1973b; Raynaud et al., 1980; Emadian and Luttge, unpublished; Luttge and Rupp, unpublished).

Because of the direct relevance of the study by Tashima et al. (1984) to our intentions of wanting to chemically eliminate Type II receptors in crude preparations, we investigated the effects of DTNB on Type I receptors in whole brain cytosol. Note that in our studies we included a 500-fold molar excess of RU 26988 to eliminate [^{3}H]ALDO cross-binding to Type II receptors. It is apparent that if DTNB is found to have no effect on Type I receptors, this reagent could be used in buffer preprations to inactivate Type II receptors selectively and completely.

In agreement with the report by Tashima et al. (1984), the addition of 1 mM DTNB to unlabeled cytosol virtually eliminated all [^{3}H]ALDO

Table 5-1. Nine parts cytosol prepared in Buffer A incubated with either one part Buffer A or adjusted to 1 mM DTNB using one part Buffer A containing 10 mM DTNB for 1 h at 0°C. Subsequently, the group that received Buffer A was diluted with either sufficient Buffer A containing 100 mM DTT to yield a final 10 mM concentration of DTT (-DTNB, +DTT) or additional Buffer A (Control; -DTNB, -DTT). The DTNB group was either brought to 10 mM concentration of DTT (+DTNB, +DTT) or diluted with an equivalent volume of 1 mM DTNB (+DTNB, -DTT). Following such treatments, all groups incubated for 24 h at 0°C with 10 nM [^{3}H]ALDO plus 5 uM RU 26988 and either with (B_{NS}) or without (B_{T}) 2 uM [^{1}H]ALDO for the determination of specific binding ($B_{SP} = B_{T} - B_{NS}$) as described in Chapter II. The data presented are from 3 independent replicate experiments and are expressed as a percent of specific binding measured in the control group $\pm$ standard error of the mean (SEM). 100% = 29 fmole/mg protein.

Table 5-1

Effects of 1 mM DTNB on Whole Brain Cytosolic Unoccupied Type I Receptors

CONDITION	PERCENT OF CONTROL $B_{SP} \pm$ SEM
Control (-DTNB, -DTT)	100 ± 0
-DTNB, +DTT	108 ± 5
+DTNB, -DTT	2 ± 1
+DTNB, +DTT	90 ± 5

binding to Type I receptors (Table 5-1). As is the case with Type II receptors, this inactivation was found to be completely reversible by replenishing the DTNB-treated cytosol with a 10-fold excess concentration of DTT. Consistent with our earlier report (Emadian et al., 1986), the addition of DTT to crude cytosol preparations had no significant effect on the binding of [^{3}H]ALDO to Type I receptors.

Effects of DTNB on Occupied Receptors.

Since DTNB and other sulfhydryl oxidizing reagents were found to act primarily on unoccupied receptors, earlier studies have implicated that these reagents inactivate Type II receptors by interacting with sulfhydryl group(s) in the steroid binding domain of these receptors (Kalimi and Love, 1980; Bodwell et al., 1984). On the basis of these reports and the fact that the equilibrium dissociation constant (K_d) of [^{3}H]ALDO from Type II receptors is about two orders of magnitude greater than that from Type I receptors (Emadian et al., 1986), we attempted to eliminate Type II receptors with DTNB in crude cytosol preparations prelabeled with [^{3}H]ALDO. Our predictions were that because of the low affinity of [^{3}H]ALDO for Type II receptors, at any given time, the chances of [^{3}H]ALDO dissociation from and subsequent inactivation of Type II receptors by DTNB would be greater than [^{3}H]ALDO reassociation with these receptors. As shown in Fig. 5-1, prolonged incubation of prelabeled cytosol with 1 mM DTNB (i.e., up to 7 h) led to a slight, but significant loss in [^{3}H]ALDO binding (in the presence and absence of RU 26988). It is noteworthy that the inactivating effects of DTNB on [^{3}H]ALDO-bound receptors were not as dramatic as those seen with unoccupied Type I (and Type II) receptors.

Figure 5-1. Effects of 1 mM DTNB on Receptors Bound to ALDO in Whole Brain Cytosol. Cytosol prepared in Buffer A was incubated with 10 nM [^{3}H]ALDO (triangles) or 10 nM [^{3}H]ALDO plus 5 uM RU 26988 (circles) and either with (B_{NS}) or without (B_T) 2 uM [^{1}H]ALDO for 48 h at 0°C. After this incubation period, each group either received sufficient Buffer A containing 10 mM DTNB to yield a final 1 mM cytosolic concentration of this compound (open trangles and circles) or diluted with an equivanlent volume of Buffer A (solid triangles and circles). Subsequently, at various time points, a fraction from each group was loaded on sephadex G-25 columns for steroid binding determination as described in Chapter II. the data presented are the mean from 3 independent replicate experiments and are expressed as a percent of control* (i.e., specific binding measured in [^{3}H]ALDO + RU 26988, -DTNB, 1 h aging group) $\pm$ standard error of the mean (SEM). 100% = 25 fmole/mg protein.

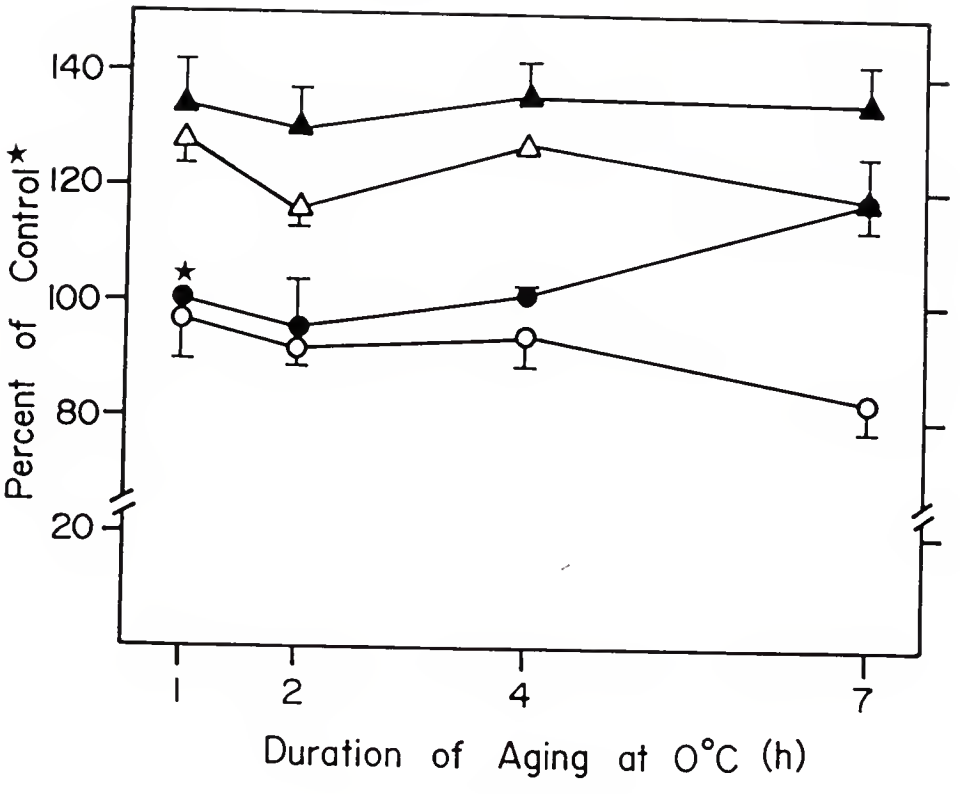
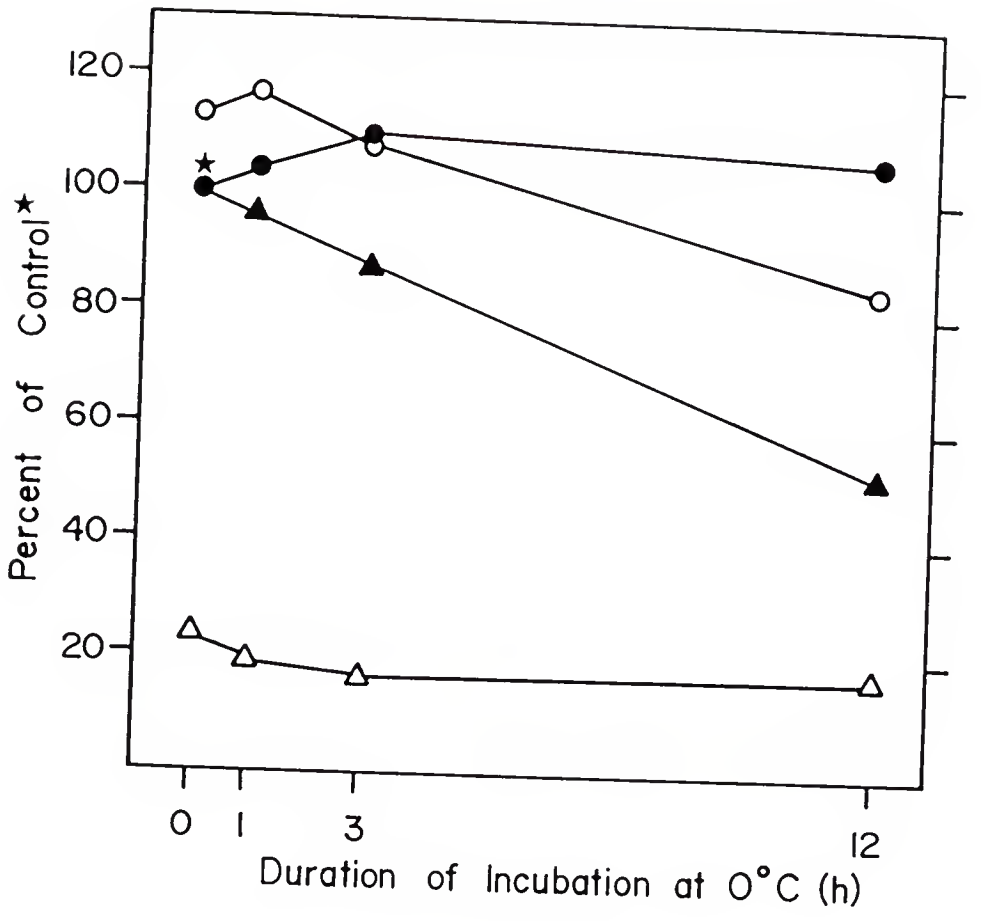

140
120
100
80
20
Percent of Control★
★
1
2
4
7
Duration of Aging at 0°C (h)

Figure 5-2. Effects of DCC Pretreatment and/or Aging of Unlabeled Whole Brain Cytosol on the Binding of ALDO to Type I and DEX to Type II Receptors. Cytosol prepared in 20 mM HEPES was either pretreated for 20 min at 0°C with DCC (0.625% dextran plus 1.25% charcoal) as described under "Materials and Methods" (open symbols) or underwent the procedure in the absence of DCC (solid symbols). Subsequently, each group was aged for 0, 1, 3 or 12 h at 0°C prior to incubation with steroid(s). Following this aging, specific Type I receptor binding was determined in the presence of 10 nM [^{3}H]ALDO plus 5 uM RU 26988 and either with (B_{NS}) or without (B_T) 2 uM [^{1}H]ALDO (circles). Type II receptor binding was determined in the presence of 20 nM [^{3}H]DEX and either with (B_{NS}) or without (B_T) 4 uM [^{1}H]DEX (triangles). The data presented are the mean from 2 independent replicate experiments and are expressed as a percent of control* (no DCC pretreatment, 0-h aging group). 100% = 23 fmole/mg protein for Type I and 341 fmol/mg protein for Type II receptors, respectively.


120
100
80
60
40
20
Percent of Control★
0
1
3
12
Duration of Incubation at 0°C (h)

Furthermore, it is apparent that with this strategy we were unable to achieve a selective Type II receptor inactivation in these preparations.

Effects of DCC Pretreatment of Cytosol on Ligand Binding Capacity of Unoccupied Type I and Type II Receptors.

As mentioned above, we reported earlier that DCC pretreatment of crude cytosol preparations inactivates the ligand binding capacity of Type II, but not Type I, receptors (Emadian et al., 1986). Since this reversible and selective inactivation appeared to be partial, in the next series of experiments we attempted to completely inactivate the remaining specific [^{3}H]DEX binding using several modified DCC pretreatment procedures.

Because Type II, but not Type I, receptors in the absence of DTT lose their ligand binding capacity in a time-dependent manner (Emadian et al., 1986; Densmore, 1986), we sought to investigate the effects of aging on the remaining [^{3}H]DEX binding in DCC pretreated cytosol preparations. It is important to emphasize that our expectations were that the DCC pretreatment procedure would further exacerbate this DTT- and time-dependent Type II receptor inactivation by removing endogenous sulfhydryl reducing agents from cytosol. Consistent with our previous report (Emadian et al., 1986), DCC pretreatment of cytosol in Buffer A (note that this buffer lacks 10% glycerol used in our earlier studies) selectively reduced the [^{3}H]DEX binding by about 80% (Fig. 5-2). Although aging the crude cytosol at 0°C produced a significant loss in specific [^{3}H]DEX binding, aging the DCC pretreated preparations for up to 12 h caused no further loss on the receptor binding capacity of this steroid. In contrast, unoccupied Type I receptors in the crude untreated, but not DCC-pretreated cytosol preparations were unaffected by aging for up to 12 h at 0°C. Parenthetically, this latter finding is

in agreement with our earlier observations that unoccupied Type I receptors in crude whole brain cytosol prepared in buffer A are fairly stable for up to 48 h at 0°C (Emadian and Luttge, unpublished, see Chapter III).

To achieve our original goal of complete and selective inactivation of Type II receptors in cytosol preparations, we next sought to investigate the effects of DCC concentration and duration of cytosol pretreatment on the inactivation of the remaining [^{3}H]DEX binders. As shown in Fig. 5-3, neither the concentration, nor the duration of treatment with a fixed concentration of DCC mixture produced further inactivating effect on the remaining [^{3}H]DEX binders. Moreover, two consecutive 20-min pretreatments of cytosol with DCC (0.1325% Dextran plus 0.625% charcoal) produced [^{3}H]DEX binding comparable to the values seen with one DCC treatment (i.e., 9 vs. 11%, respectively) (Table 5-2). It is noteworthy that an increase in the concentration, duration and the number of DCC treatments significantly reduced the [^{3}H]DEX binding that could be recovered by adding 2 mM DTT and 20 mM molybdate to the DCC pretreated preparations.

Because, 1) the specific [^{3}H]DEX binding remaining after DCC pretreatment of cytosol is approximately 1/10 of total [^{3}H]DEX binding in crude cytosol prepared in Buffer DM (an optimal buffer for Type II receptors to bind ligand), 2) the concentration of Type I receptors in whole brain cytosol preparations on a fmole/mg protein basis is approximately 1/10 of the Type II receptors in the same preparations (Wrange and Yu, 1983; Emadian et al., 1986), 3) Type I receptors are unaffected by the DCC pretreatment procedures and 4) there is a small, but significant [^{3}H]DEX cross-binding with Type I

Figure 5-3. Effects of Pretreatment of Whole Brain Cytosol with Various Concentrations of DCC and Different Duration of DCC Pretreatment on the Binding of DEX to Type II Receptors. Cytosol prepared in 20 mM HEPES buffer was either (a) pretreated for 20 min at 0°C with 1/8, 1/4, 1/2, 1, 2 or 4-fold concentration of DCC used in Fig. 5-2 (i.e., 0.625% Dextran to 1.25% charcoal); (b) pretreated with 0.3125% dextran and 0.625% charcoal mixture from 10 to 120 min at 0°C as described under "Materials and Methods" (the 1/2 group) or (c) underwent the procedure in the absence of DCC (the 0 group). Subsequently, 9 parts of each group was diluted either with one part 20 mM HEPES buffer containing 20 mM DTT and 200 mM molybdate to yield a final concentration of 2 mM DTT and 20 mM molybdate in cytosol (open and hash marked bars) or diluted appropriately with one part 20 mM HEPES buffer (solid bars). Following these steps, all groups were incubated for 24 h at 0°C with 20 nM [^{3}H]DEX and either with (B_{NS}) or without (B_{T}) 4 uM [^{1}H]DEX. The data presented are the mean from at least 2 independent experiments and are expressed as a percent of control* (open bar). 100% = 427 fmol/mg protein.

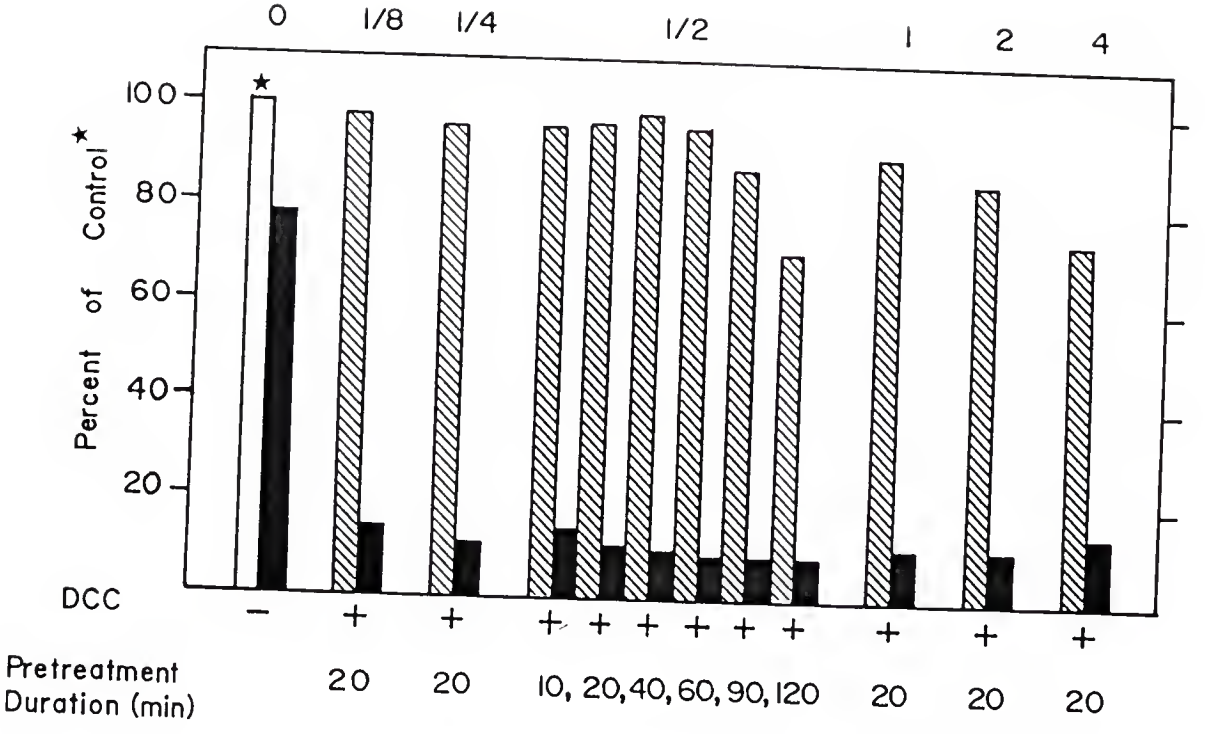

0
1/8
1/4
1/2
1
2
4
100
80
60
40
20
Percent of Control*
DCC
−
+
+
+
+
+
+
+
+
+
+
+
Pretreatment
Duration (min)
20
20
10, 20, 40, 60, 90, 120
20
20
20

Table 5-2. Cytosol prepared in 20 mM HEPES buffer was pretreated once (+DCC) or twice (+2x DCC) consecutively for 20 min each time at 0°C with DCC (i.e., 0.3125% Dextran to 0.625% charcoal) as described under "Materials and methods," or underwent the procedure in the absence of DCC (-DCC). Subsequently, 9 parts of each group was diluted either with one part 20 mM HEPES buffer containing 20 mM DTT and 200 mM molybdate to yield the composition of Buffer DM_{20} (+DM) or diluted appropriately with one part 20 mM HEPES buffer (-DM). Following these steps, all groups were incubated for 24 h at 0°C with 20 nM [^{3}H]DEX and either with (B_{NS}) or without (B_T) 4 uM [^{1}H]DEX. The data presented are the mean from at least 2 independent replicate experiments and are expressed as a percent of control (specific [^{3}H]DEX binding in -DCC, +DM group) $\pm$ standard error of the mean (SEM) when the mean is from at least 3 independent replicate experiments. 100% = 427 fmole/mg protein.

Table 5-2

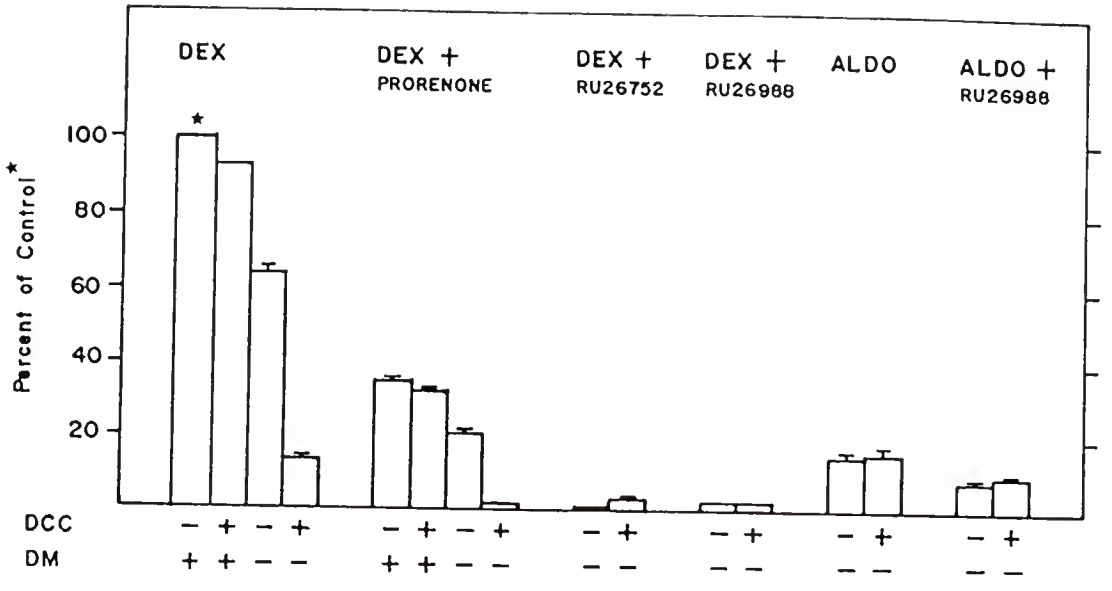
Effects of Two Consecutive DCC Pretreatments of Cytosol on the Residual DEX Specific Binding

CONDITION	PERCENT OF CONTROL $B_{SP} \pm$ SEM
Control (-DCC, +DM)	100 ± 0
-DCC, -DM	70 ± 2
+DCC, -DM	11
+DCC, +DM	97
+2x DCC, -DM	9 ± 1
+2x DCC, +DM	87 ± 1

receptors (Raynaud et al., 1980), we found it essential to eliminate the possibility that the remaining [^{3}H]DEX binding following DCC pretreatment represent binding of this ligand to Type I receptors.

In the next experiment, we measured Type I and Type II receptor binding in DCC pretreated preparations in the presence and absence of a "specific" Type I and Type II receptor blocker; i.e., prorenone and RU 26752 (antimineralocorticoid spironolactones) and RU 26988 (a specific antiglucocorticoid), respectively, in conjunction with [^{3}H]ALDO and [^{3}H]DEX. Despite the reported specificity of RU 26752 and prorenone for Type I receptors (e.g., Claire et al., 1979; Coirini et al., 1985), the introduction of a 500-fold molar excess of prorenone RU 26752 (Fig. 5-4) in incubation media produced an 80% and 99% reduction (-DCC, -DM) in the measurable [^{3}H]DEX binding, respectively. Furthermore, even in the presence of prorenone, there was a small but rather significant residual [^{3}H]DEX binding after pretreatment of cytosol with DCC (+DCC, -DM). As shown in Fig. 5-4, the magnitude of [^{3}H]DEX binding prior to as well as following DCC pretreatment was the same when the incubation media contained a 500-fold molar excess of RU 26988 and RU 26752. By comparison, in the absence of RU 26988, there was a 50% increase in the measurable specific [^{3}H]ALDO binding (i.e., [^{3}H]ALDO binding in the absence of RU 26988 was about 17% of total [^{3}H]DEX binding, whereas that seen in the presence of this Type II receptor blocker was about 9%) (Fig. 5-4). As a caution, it is important to note that although these synthetic steroids may manifest <u>biological specificity</u> for the action of an endogenous steroid, one cannot conclude <u>binding specificity</u> for the receptors through which the action(s) of these endogenous steroids are presumably mediated.

Figure 5-4. Effects of DCC Pretreatment of Whole Brain Cytosol on the Binding of ALDO and DEX to Type I and Type II Receptors in the Presence or Absence of a Steroid Competitor. Cytosol prepared in 20 mM HEPES was either pretreated for 20 min at 0°C with DCC (0.1325% dextran to 0.625% charcoal) as described under "Materials and Methods" (+DCC) or underwent the procedure in the absence of DCC (-DCC). Subsequently, nine parts cytosol from each group was diluted with either one part 20 mM HEPES buffer containing 20 mM DTT and 200 mM molybdate to yield a final concentration of 2 mM DTT and 20 mM molybdate in cytosol (+DM) or diluted appropriately with one part 20 mM HEPES buffer (-DM). Following such treatments, each group was incubated with the following steroid(s) for 24 h at 0°C prior to specific binding determination. a) 20 nM [^{3}H]DEX, b) 20 nM [^{3}H]DEX + 10 uM Prorenone, c) 20 nM [^{3}H]DEX + 10 uM RU 26752, d) 20 nM[^{3}H]DEX + 10 uM RU 26988, e) 10 nM [^{3}H]ALDO and f) Open bars: 10 nM [^{3}H]ALDO + 5 uM RU 26988. Non-specific binding for [^{3}H]ALDO and [^{3}H]DEX was determined in the presence of 2 uM [^{1}H]ALDO and 4 uM [^{1}H]DEX, respectively. The data presented are the mean from 3 independent replicate experiments and are expressed as a percent of control* ([^{3}H]DEX binding in -DCC, +DM group) $\pm$ standard error of the mean (SEM). 100% = 460 fmole/mg protein.


DEX
DEX +
PRORENONE
DEX +
RU26752
DEX +
RU26988
ALDO
ALDO +
RU26988
Percent of Control*
100
80
60
40
20
DCC
DM
− + − +
+ + − −
− + − +
+ + − −
− +
− −
− +
− −
− +
− −
− +
− −

Since the inactivating effects of DCC on Type II receptors was partial, and because 300 mM KCl was found to produce a time-dependent loss in the binding capacity of unoccupied Type II, but not Type I receptors (Fig. 5-5; Densmore et al. 1984a), we next attempted to inactivate the residual 10-20% [^{3}H]DEX binding by incubating DCC pretreated cytosol preparations in the presence of 300 mM KCl. Parenthetically, it is interesting to note that although 20 mM molybdate leads to significant reduction in the subsequent binding of [^{3}H]ALDO to Type I receptors (Fig. 5-5, KM; also see Fig. 4-2 and Emadian et al., 1986), in the presence of 300 mM KCl, the magnitude of specific [^{3}H]ALDO binding is even higher than the level seen in the absence of these buffer components (Fig. 5-5, A). It is entirely possible that 300 mM KCl in conjunction with 20 mM molybdate induce a conformation of Type I receptors that is optimal for ligand binding. This binding increase is also seen in the presence of 10% ethanol (Chapter III) and 2 mM molybdate in gel filtered cytosol preparations (Chapter IV).

As shown in Fig. 5-6, 300 mM KCl was found to be ineffective in further eliminating the [^{3}H]DEX binders resistant to the inactivating effects of DCC. Note that DCC and/or 300 mM KCl significantly reduced the binding of [^{3}H]ALDO in the absence, but not presence of RU 26988. Therefore, it appears that a significant proportion of [^{3}H]ALDO binding in untreated cytosol in the absence of RU 26988 is to Type II receptors sensitive to the inactivating effects of DCC and KCl (i.e., the classical Type II receptors).

A possible mechanism by which KCl inactivates unoccupied Type II receptors may be through subunit dissociation of the multimeric form of the receptors; i.e., by generating the 92 Kda steroid-binding subunit

Figure 5-5. Effects of 300 mM KCl in the Presence and/or Absence of 20 mM Molybdate on the Binding of ALDO to Type I and DEX to Type II Receptors. Nine parts cytosol prepared in Buffer A was diluted either with one part 20 mM HEPES buffer containing 200 mM molybdate (M), 3 M KCl (K), 200 mM molybdate plus 3 M KCl (KM) or 20 mM HEPES buffer (A) to yield the concentration of the desired component(s) in cytosol. Subsequently, each group either incubated with steroid(s) for 24 h at 0°C or aged at 0°C from 2 to 6 h prior to incubation with steroid(s). Circles: Type I receptor binding was determined in the presence of 10 nM [^{3}H]ALDO and 5 uM RU 26988 (B_T). Triangles: Type II receptor binding was determined using 20 nM [^{3}H]DEX (B_T). Non-specific binding for Type I and Type II receptors was determined in the presence of 2 uM [^{1}H]ALDO and 4 uM [^{1}H]DEX, respectively. The data presented are the mean from 4 independent replicate experiments and are expressed as a percent of control* (receptor binding in non-aged cytosol in Buffer A) $\pm$ standard error of the mean (SEM). 100% for Type I receptors = 23 fmol/mg protein and 100% for Type II receptors = 275 fmol/mg protein.

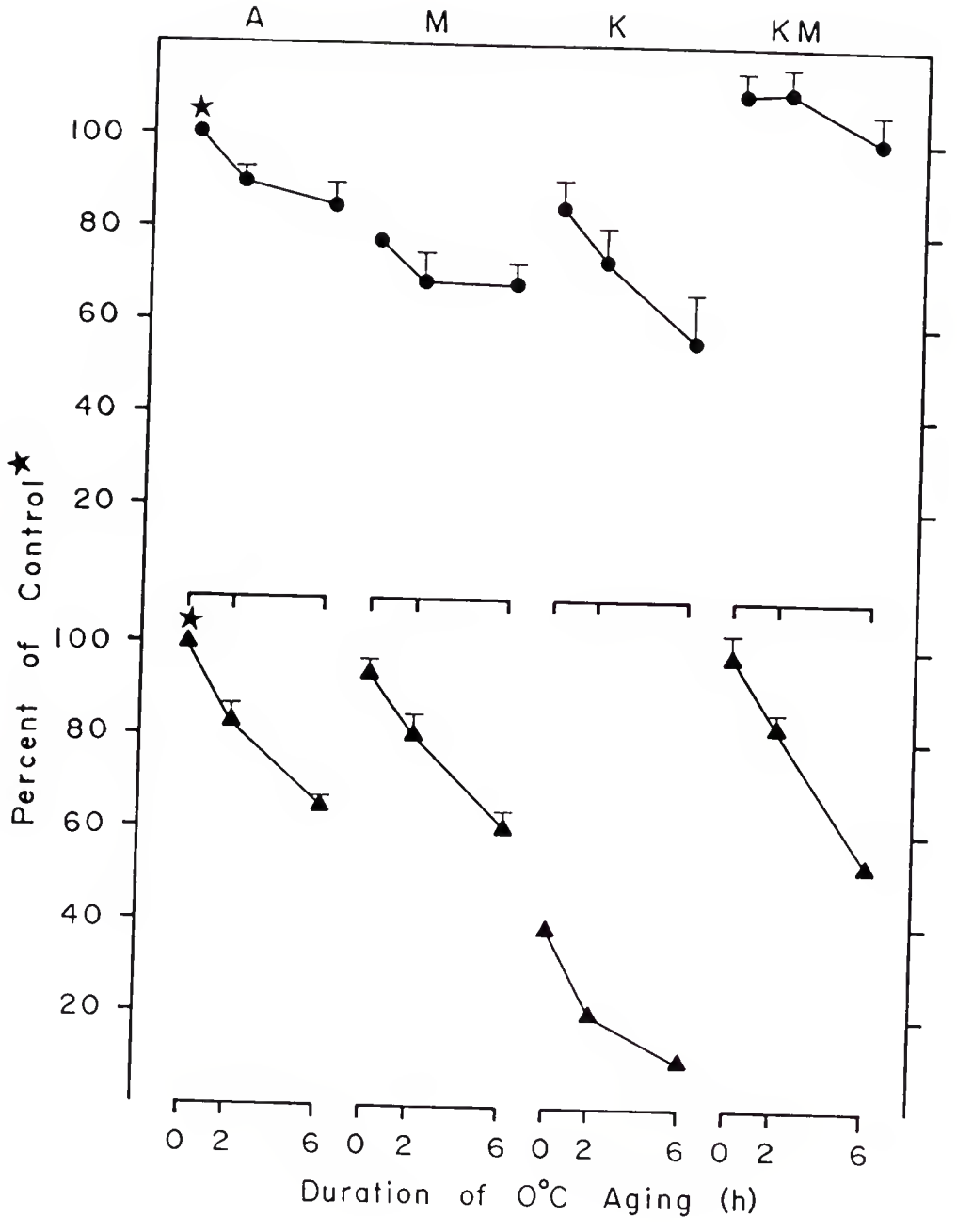
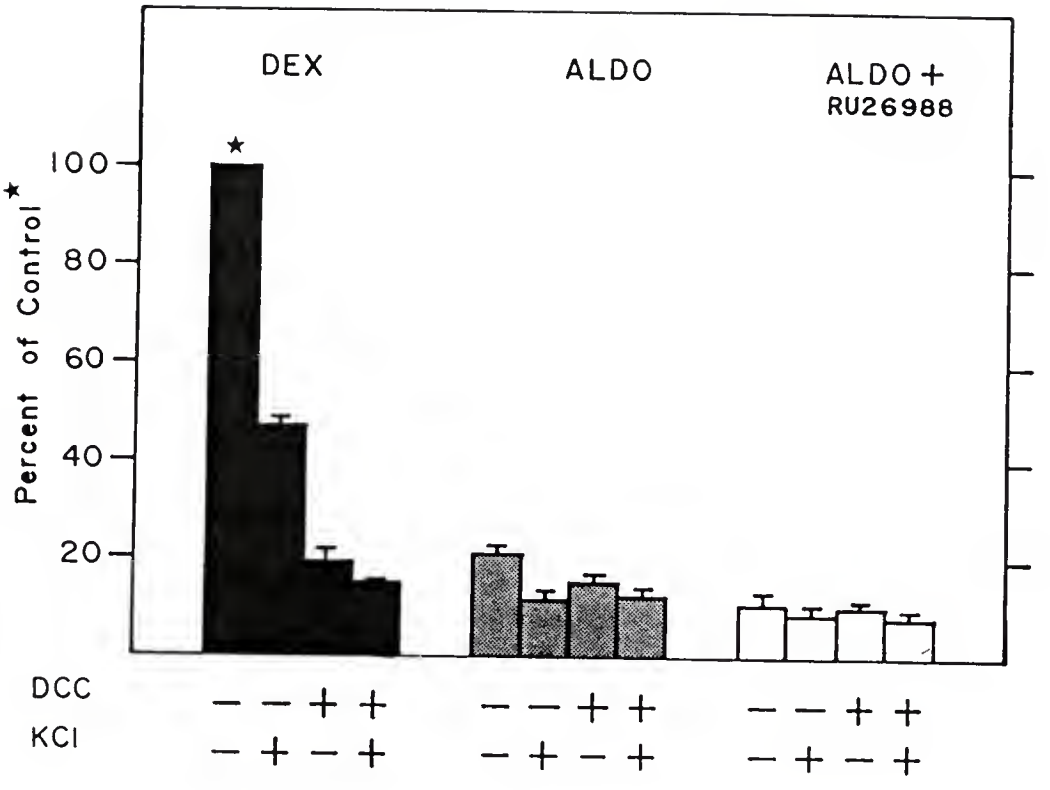

A
M
K
KM
100
80
60
40
20
Percent of Control★
0 2 6 0 2 6 0 2 6 0 2 6
Duration of 0°C Aging (h)

Figure 5-6. Effects of 300 mM KCl on the Specific Binding of ALDO and DEX Prior and Subsequent to DCC Pretreatment. Cytosol prepared in 20 mM HEPES buffer was either treated with DCC (+DCC), or underwent the procedure in the absence of DCC (-DCC) as described under "Materials and Methods." Subsequently, nine volumes of DCC- or non-DCC-treated cytosol was diluted with either one volume of 3 M KCl (+KCl) or one volume of 20 mM HEPES buffer (-KCl). Following such treatments, each group was incubated with the following steroid(s) for 24 h at 0°C: a) 20 nM [^{3}H]DEX (solid bars), b) 10 nM [^{3}H]ALDO (shaded bars) and c) 20 nM [^{3}H]ALDO + 5 uM RU 26988 (open bars). Non-specific binding for [^{3}H]ALDO and [^{3}H]DEX was determined in the presence of 2 uM [^{1}H]ALDO and 4 uM [^{1}H]DEX, respectively. The data presented are the mean from 5 independent replicate experiments and are expressed as a percent of control* (i.e., specific [^{3}H]DEX binding measured in -DCC and -KCl group) $\pm$ standard error of the mean (SEM). 100% = 315 fmol/mg protein.


DEX
ALDO
ALDO +
RU26988
Percent of Control*
100
80
60
40
20
DCC
KCl
− − + +
− + − +
− − + +
− + − +
− − + +
− + − +

(the "activated" receptor) (for detail se Chapter VI). Note that the results from this and other laboratories suggest that the activated Type II receptors in crude cytosol preparations are unable to rebind glucocorticoids following ligand dissociation (Chou and Luttge, 1987). The idea that KCl may inactivate Type II receptors through subunit dissociation is further supported by the fact that molybdate, which prevents the salt induced activation of occupied Type II receptors (see Chapter VI), is also capable of blocking the inactivating effects of KCl on unoccupied Type II receptors (Fig. 5-5, KM).

Collectively, these data suggests that the [^{3}H]DEX binding (and some of [^{3}H]ALDO binding in the absence of RU 26988) remaining after DCC pretreatment of cytosol represents, at least in part, ligand binding to a new class of Type II receptors with chemical properties distinct from the classical Type II receptors. Further evidence for this claim comes from a close examination of the data presented in Fig. 5-2. Whereas the receptors from DCC pretreated cytosol that bound [^{3}H]DEX were unaffected by aging the unlabeled cytosol at 0°C, Type I receptors (i.e., [^{3}H]ALDO binding in the presence of RU 26988) in the same preparations appeared to lose their ligand binding capacity in a time-dependent manner at 0°C.

In conclusion, aside from the potential methodological implications, the data presented here provide a number of suggestions regarding the <u>in vivo</u> regulation of Type I and Type II receptors. For example, it is apparent that the factors that are removed from cytosol by DCC pretreatment and which have been implicated in the <u>in vivo</u> regulation of Type II receptors (e.g., thioredoxin and NADPH) (Grippo et al., 1983 & 1985), may not be involved in the

regulation of Type I receptors or the new class of Type II receptors discussed above. In other words, there appears to be at least two separate populations of [^{3}H]DEX binders in brain. One population of these binders is readily susceptible to up- and down-regulation by sulfhydryl oxidation-reduction cycle, while a second population is immune from this regulatory mechanism and may provide a constitutive level of viable receptors even under challenging conditions. As an example of one possible expression of this relationship, recent experiments from our laboratory investigating the ligand-induced in vivo regulation of Type I and Type II receptors in the brain, kidney and liver suggest that treating mice even with pharmacological doses of corticosterone fails to produce a complete down-regulation of Type II receptors in these tissues (Luttge and Rupp, unpublished). It is entirely possible that the population of [^{3}H]DEX binders remaining after corticosterone treatment is enriched in the new class of Type II receptors shown here to be resistant to the DCC pretreatment, absence of DTT, high ionic strength and aging at 0°C. Investigation of the above possibility as well as further physicochemical characterization of this new class of Type II receptors is currently under way in our laboratory.

CHAPTER VI
IN VITRO TRANSFORMATION OF ALDOSTERONE-TYPE I RECEPTOR COMPLEXES TO A DNA-BINDING STATE

Summary

In order to modulate DNA transcription, steroid-receptor complexes are thought to undergo a "transformation" (activation) process that increases the affinity of the complexes for specific regions of DNA called hormone response elements. It has been shown that once activated androgen-, estrogen-, progesterone- and glucocorticoid-receptor complexes attain physicochemical properties that are distinct from those of the unactivated steroid-receptor complexes. In this chapter, an investigation of the properties of ALDO-Type I receptors prior and subsequent to exposure of brain cytosol to 300 mM KCl and/or 22°C for 20 min (conditions known to result in complete activation of glucocorticoid-Type II and other steroid-receptor complexes) was conducted. Exposure to 300 mM KCl and 22°C for 20 min, led to a 3-fold increase in the binding of ALDO-Type I receptors to DNA-cellulose (DNA-C) (6 and 19% binding for unactivated and activated receptors, respectively). In contrast, identical experimental conditions produced a 14-fold increase in the binding of Type II-receptor complexes to DNA-C (4 and 54% binding for unactivated and activated receptors, respectively). Molybdate, a known inhibitor of activation of all steroid-receptor complexes, also prevented this salt- and temperature-induced increase in the binding of ALDO-Type I receptors to DNA-C. Dithiothreitol was found to have no effect on the binding of activated ALDO-Type I and DEX-Type II receptors to DNA-C; however, the

presence of 5 mM molybdate in buffers during DNA-C batch assay produced the largest difference in the magnitude of DNA-C binding between unactivated and activated ALDO-Type I receptor complexes (i.e., 6 versus 20% in the presence and 13 versus 14% in the absence of this oxyanion). Although previous work from our laboratory showed that activation of glucocorticoid-Type II receptor complexes is followed by a marked change in surface hydrophobic properties of these receptors, salt and heat produced only subtle changes in surface hydrophobicity of ALDO-Type I receptor complexes. Taken together, these results suggest that conditions leading to complete activation of Type II and other steroid receptors will only produce partial activation of ALDO-Type I receptor complexes.

Introduction

Although the exact *in vivo* mechanisms or "factors" that bring about activation of steroid-receptor complexes remain to be elucidated, a number of *in vitro* manipulations are known to stimulate activation. For example, *in vitro* activation of glucocorticoid-Type II receptors can be triggered by increasing cytosolic ionic strength and/or temperature (Higgins et al., 1973; Kalimi et al., 1975; Muller et al., 1983; Luttge and Densmore, 1984; Luttge et al., 1984a, b & d), gel filtration (Cake et al., 1976; Bailly et al.; 1977 & 1978; Distelhorst and Benutto, 1984), dilution (Higgins et al., 1973; Goidl et al., 1976), increase in pH (Bailly et al., 1978) and by addition of a variety of chemicals such as ATP (Moudgil and John, 1980) and heparin (McBlain and Shyamala, 1980; Yang et al., 1982; Hubbard and Kalimi, 1983). The involvement of cysteine and serine proteases have also been implicated in the activation process (Hubbard and Kalimi, 1985).

Regardless of the factors eliciting activation, a number of physicochemical changes have been shown to characterize this transformation. For example, it has been demonstrated that the activation of glucocorticoid-Type II receptor complexes is accompanied by a conformational change resulting in the exposure of additional positive charges on the surface of the complex (Milgrom et al., 1973a; Luttge et al., 1984a & d). This increase in surface positivity can be demonstrated by a reduction in affinity of the activated complexes for diethylaminoethyl-cellulose (DEAE-cellulose) columns or filters (Sakaue and Thompson, 1977), and an increase in the affinity for nuclei, DNA, and other polyanions (Alberts and Herrick, 1971; Kalimi et al., 1975; Luttge et al., 1984a, c & d). Activation is also characterized by an increase in the overall surface hydrophobicity of the steroid-receptor complexes. This increase in hydrophobicity (which may facilitate nuclear membrane penetration and/or interaction with specific hormone response elements on the DNA molecule) has been measured by an increase in the binding of the complexes to glass-fiber filters (GF/C) (Luttge et al., 1984b & d), aqueous dextran-polyethylene glycol two-phase partitioning (Andreasen, 1982; Luttge et al., 1984d) and hydrophobic interaction chromatographic techniques (Bruchovsky et al., 1981; Geschwendt and Kittstein, 1980; Densmore, 1986; Densmore et al., 1986b). Moreover, activation of a number of steroid-receptor systems has been shown to be accompanied by a reduction in the rate of steroid dissociation (e.g., McBlain et al., 1981; Moguilewsky and Philibert, 1984; Chou and Luttge, 1987).

Earlier studies regarding such alterations in the hydrodynamic properties of the steroid-receptor complexes following activation

appeared inconsistent. In many cases, these inconsistencies were primarily due to a failure to control spontaneous activation and receptor degradation during prolonged ultracentrifugation and open column analytical gel exclusion chromatographic procedures. Note that the in vitro activation of a number of steroid-receptor complexes can slowly occur even at near zero temperatures (Luttge and Densmore, 1984). The utilization of molybdate to inhibit activation during cytosol preparations, labeling and prolonged hydrodynamic parameter determination has allowed the generation of more consistent results (e.g., Dahmer et al., 1981; Luttge and Densmore, 1984; Luttge et al., 1984a, b & d). For example, our laboratory has reported that activation is accompanied by a reproducible reduction in the sedimentation coefficient of glucocorticoid-Type II receptor complexes (form 9.2 S to a 3.8 S) (Luttge and Densmore, 1984; Luttge et al., 1984a, b & d). This reduction in sedimentation coefficient is also accompanied by a reduction in the Stoke's radius (from 7.7 nm to 5.8 nm) and the relative molecular mass (from 297 kDa to 92 kDa) of the glucocorticoid-receptor complexes (Luttge et al., 1984a & d).

Although activation of steroid-receptor complexes is thought to be a prerequisite step for genomic response, the exact nature of the nuclear "acceptor" sites through which the complexes modulate cellular events remained an enigma for some time. Hormone-receptor complexes were shown to interact and be associated with a variety of partially purified nuclear components including DNA (Higgins et al., 1973; Tymoczko et al., 1984), RNA (Feldman et al., 1981; Rossini and Barbiroli, 1983; Tymoczko and Phillips, 1983; Economidis and Rosseau, 1985; Reker et al., 1985; Tymoczko and Lee, 1985; Webb et al., 1986;

Ali and Vedeckis, 1987) and histones (Kallos et al., 1981). In addition, it is becoming increasingly apparent that a salt- and nuclease-resistant nuclear substructure, known as the "nuclear matrix," may play an important role in the genomic expression of hormone-receptor action (for review see Barrack and Coffey, 1982; Capco et al., 1982).

The nuclear matrix (NM) was shown to be enriched with newly replicated DNA and heterogeneous nuclear RNA (hn-RNA) (Berezney and Buckholtz, 1981; Ciejek et al., 1982; for review see Nelson et al., 1986). These properties of NM, as well as the observations that in target tissues high-affinity steroid binding sites are associated with NM (Barrack and Coffey, 1980 & 1982; Barrack, 1983; Spelsberg et al., 1983), were taken as evidence for NM as the site for steroid-receptor complex modulation of mRNA transcription (Barrack and Coffey, 1982; Buttyan et al., 1983; Simmen et al., 1984; Kirsh et al., 1986). Interestingly, it appears that different steroid-receptor complexes bind preferentially to the NM of their respective target tissues (Buttyan et al., 1983). Partial extraction of the DNA associated with NM resulted in a loss in the specificity of the NM-steroid-receptor complex interactions. It is thus proposed that the DNA associated with the NM of target tissues may, at least in part, confer this binding specificity (Buttyan et al., 1983).

DNA-cellulose competition assays have also been used to show that activated steroid-receptor complexes manifest stereospecific binding to DNAs containing specific sequences. For example, purified chick oviduct progesterone-receptor complex was found to bind preferentially to a 1.7 kilobase DNA fragment flanking the 5' end of the chicken ovalbumin gene with A--T rich base pair sequences (Hughes et al., 1981;

Compton et al., 1982; Mulvihill et al., 1982; Compton et al., 1983). Activated glucocorticoid-Type II receptor complexes were shown to interact with high affinity at the promotor region of the mouse mammary tumor (MMTV) proviral DNA (Pfahl, 1982). Recently, purified steroid hormone receptors were shown to bind specifically to palindromic sequences within the DNA molecule in order to modulate the transcription of mRNAs (e.g., Wrange et al., 1986; Arneman et al., 1987; Chambone et al., 1987; Drouin et al., 1987; Karin et al., 1987; Meisfeld et al., 1987). Together, these observations suggest that activated steroid-receptor complexes may act in a manner comparable to other DNA regulatory proteins such that by interaction with specific sites on the DNA molecule they may modulate the activity of adjacent promotor regions, and hence selectively modulate the transcription of specific DNA transcripts.

Despite the wealth of our knowledge regarding the physicochemical changes accompanying activation of Type II, progesterone, estrogen and androgen receptors, the existing information on the activation of Type I receptors is limited and in most instances difficult to interpret. In earlier studies, a major difficulty associated with the interpretation of the results is the confounding effects of ALDO binding to Type II receptors (Marver et al., 1972; Kolpakov et al., 1974; Kusch et al., 1978; Farman et al., 1981; Palem-Vliers et al., 1982; Lehoux et al., 1984). Despite the lack of direct evidence regarding the activation of Type I receptors in the studies cited above, indirect evidence clearly favored the interaction of ALDO-receptor complexes with DNA. For example, in addition to inhibiting the physiological action of ALDO, certain spironolactones were shown to produce a dose-dependent reduction

in in vitro nuclear uptake of [^{3}H]ALDO (Marver et al., 1972) and chromatin binding (Swaneck et al., 1970). Furthermore, various DNA intercalators (e.g., actinomycin D, ethidium bromide, netropsin and proflavin sulfate) were shown to inhibit the binding of [^{3}H]ALDO-receptor complexes to chromatin extracts, thus ultimately inhibiting the synthesis of AIPs (Edelman et al., 1963; Edelman and Marver, 1980). The degree of this inhibition appeared to be related to specificity and concentration of the intercalator used, hence implicating DNA as a candidate for complex formation (Edelman and Marver, 1980). Lastly, site-specific and saturable nuclear [^{3}H]ALDO binding in a number of target tissues including the brain (Ermisch and Ruhle, 1978; Birmingham et al., 1984), kidney (Vandewalle et al., 1981; Farman et al., 1981, 1982a & b; Farman and Bonvalet, 1983) and toad urinary bladder (Farman et al., 1978) was detected by autoradiographic and other biochemical techniques.

Recently, two independent studies on activation of rat kidney [^{3}H]ALDO-Type I receptors (note that ALDO binding to Type II receptors was prevented by including a molar excess of a glucocorticoid competitor in the incubation media) have expressed difficulties in achieving full activation of these receptors (measured by DEAE-cellulose, Sephacryl S-300 and DNA-C column chromatographic techniques) (Eisen and Harmon, 1986; Schulman et al., 1986). The results from the experiments described in this chapter also suggest that conditions resulting in full activation of brain DEX-Type II receptors produce only partial activation of ALDO-Type I receptor complexes.

Materials and Methods

Chemicals. Double stranded calf thymus DNA-cellulose (DNA-C) (5.7 mg DNA/g DNA-C) and PMSF were purchased from Sigma. All other chemicals used in this chapter were reagent grade quality.

Buffers. Buffer K contained 20 mM HEPES and 300 mM KCl, Buffer DK was buffer K plus 2 mM DTT, Buffer KM was Buffer K plus 20 mM molybdate and Buffer DMK contained all of the above constituents. Buffer M_5 contained 20 mM HEPES and 5 mM molybdate. In some experiments, the cytosolic concentration of Buffers K, DK, KM and DKM were obtained by diluting 9 parts cytosol with one part buffer containing a 10-fold concentration of the desired buffer. Alternatively, cytosol preparations were eluted from Sephadex G-25 columns pre-equilibrated with the desired buffer to bring the preparations to the composition of that buffer (see specific experiments). All buffers were adjusted to pH 7.60 just prior to use.

Activation. Cytosol was incubated with steroid(s) at 0°C for 24 to 48 h to label Type I or Type II receptors (for detail see Chapter II). Prior to initiation of activation, free steroid(s) were removed by gel exclusion chromatography on Sephadex G-25 columns (0.6 x 14.0 cm) pre-equilibrated in Buffer K, DK, KM or DMK (see specific experiments) as described in Chapter II. To activate the steroid-receptor complexes, samples obtained in the void volume were placed for 20 min in a water bath maintained at 22°C. Activation was quenched by returning the samples to ice bath followed by a second Sephadex G-25 gel filtration to bring the preparations to buffer formulation desired and to remove steroids dissociated during activation process.

DNA-C Binding Assay. Double stranded calf thymus DNA-C was prepared overnight in Buffer A or Buffer A containing other component(s) (see specific experiments) at a final concentration of 10 mg/ml. Just prior to use, 0.8 ml aliquots of the slurry were transferred to separate test tubes, the DNA-C pelleted by centrifugation at 2,280 x g in a model RC-3 Sorvall centrifuge for 10 min and the supernatant was aspirated carefully and discarded. Subsequently, 0.8 ml samples of cytosol were added to the DNA-C pellet. The DNA-C was then resuspended by gentle vortexing and incubated in ice bath for 60 min. The assay tubes were oscillated at 150 RPM throughout this incubation period and vortexed gently every 10 to 15 min. At the end of the incubation period, steroid-receptor complex bound to DNA-C was collected by centrifugation at 2,280 x g for 10 min, the supernatant aspirated and the DNA-C pellet washed twice with 1.0 ml buffer identical in composition to the original cytosolic buffer. The DNA-C was then transferred quantitatively to scintillation vials for determination of bound radioactivity as described in Chapter II.

Results

As shown in Table 6-1, unactivated ALDO-Type I and DEX-Type II receptor complexes show minimal affinity for DNA-C (i.e., 6 and 4% when maintained at 0°C in Buffer DMK, respectively). Whereas heating DEX-Type II receptors in Buffer K or DK for 20 min at 22°C produced a 14-fold increase in the binding of these complexes to DNA-C (i.e, 54% binding to DNA-C), under identical conditions, DNA-C binding for ALDO-Type I receptor complexes did not exceed a 3-fold rise (i.e., 19% binding to DNA-C in Buffer DK). Thus far, several other manipulations (e.g., increasing the temperature and/or duration of activation) failed

Table 6-1. Nine parts cytosol prepared in Buffer M_{20} was diluted either with one part Buffer M_{20} containing 20 mM DTT or Buffer M_{20} alone to yield cytosol with composition of Buffer DM_{20} or M_{20}, respectively. Type I receptors were then labeled with 10 nM [^{3}H]ALDO in the presence of 5 uM RU 26988 and Type II receptors were labeled with 10 nM [^{3}H]DEX. B_{NS} determinations for Type I and Type II receptors were in the presence of 2 uM [^{1}H]ALDO and [^{1}H]DEX, respectively. Following a 24-h incubation at 0°C with the steroids, 0.5 ml fractions were loaded on Sephadex G-25 columns (pre-equilibrated with Buffer K, KM, DK or DMK) to remove free steroids (and molybdate under Buffer K and DK conditions) and to bring the cytosol to the composition of the desired Buffers (i.e.; K, KM, DK or DMK). Subsequently, sufficient 100 mM phenylmethyl sulfonylfluoride (PMSF) dissolved in absolute ethanol was added to cytosol to yield a final 1 mM concentration of this compound. Activation was then performed as described under "Materials and Methods" and DNA-C binding assays were performed in the presence of Buffer DM_5 as described under "Materials and Methods."

Table 6-1

Comparison of the Binding of ALDO-Type I and DEX-Type II Receptor Complexes to DNA-Cellulose Before and After Salt- and/or Temperature-Induced Activation

BUFFER	TEMPERATURE			
	0°C		22°C	
	Type I	Type II	Type I	Type II
DMK	6 ± 1	4 ± 1	7 ± 2	9 ± 1
DK	8 ± 2	41 ± 2	19 ± 1	54 ± 1
KM	8 ± 2	7 ± 1	8 ± 1	11 ± 0
K	7 ± 1	35 ± 2	16 ± 1	54 ± 3

to produce DNA-C binding of ALDO-Type I receptor complexes greater than 20% (data not shown). Although 300 mM KCl in the absence of heat was able to increase DNA-C binding of DEX-Type II receptor complexes significantly (i.e., 41 and 35% in Buffer DK and K, respectively), the magnitude of the DNA-C binding of ALDO-Type I receptor complexes maintaied at 0°C ranged from 6 to 8% under all buffer conditions studied. As with other steroid hormone receptor systems, inclusion of 20 mM molybdate (a group VI transition metal oxyanion) prevented the increases in DNA-C binding of ALDO-Type I and DEX-Type II receptors following activation.

Recently, structural analysis of the amino acid sequence of the steroid-binding subunit of purified Type II receptors has shown this subunit to contain a cysteine rich region in the DNA-binding domain (e.g., Danielsen et al., 1987). Earlier biochemical studies suggested that the binding of activated glucocorticoid-Type II receptor complexes to DNA required reduction of sulfhydryl groups in the DNA-binding region (Kalimi and Love, 1980; Bodwell et al., 1984). However, the study presented in Table 6-1 shows that DNA-C binding of salt- and heat-activated DEX-Type II receptor complexes is identical in the presence (Buffer DK) or absence (Buffer K) of the sulfhydryl reducing reagent, DTT. In contrast, when maintained at 0°C, the DNA-C binding of DEX-Type II receptor complexes in Buffer DK was slightly higher than that seen in Buffer K (41 versus 35%, respectively). The reason for this difference is unclear at this stage.

Since earlier experiments with Type II receptors suggested that the presence of high concentrations of molybdate can prevent the binding of activated Type II receptors to DNA-C (Luttge, unpublished results), and

because 5 mM molybdate was used during DNA-C binding to prevent spontaneous activation, the effects of this reagent on the binding of activated and unactivated [^{3}H]ALDO-Type I receptor complexes to DNA-C was next investigated. As shown in Table 6-2, the presence of 5 mM molybdate (Buffer M_5) during DNA-C binding assay produced a larger difference in the magnitude of the binding of unactivated (0°C) and activated (22°C) Type I receptors (6 versus 20% DNA-C binding, respectively) than that seen in the absence of molybdate (Buffer A) (13 versus 14% for unactivated and activated receptors, respectively). Similar results were also obtained with Type II receptors: the magnitude of difference between DNA-C binding of unactivated and activated Type II receptors was greatest in the presence of 5 mM molybdate (Luttge, unpublished results).

In addition to the increase in affinity for DNA-C following activation, a recent extensive analysis of the surface hydrophobic properties of activated and unactivated Type II receptors by hydrophobic interaction chromatography showed that the activation of these receptors is accompanied by an increase in the overall surface hydrophobicity of these receptors (Densmore, 1986). In contrast, an examination of the elution profile of ALDO-Type I receptors prior and subsequent to attempted salt- and heat-induced activation from pently-Agarose columns revealed that there were only slight differences between the unactivated and "activated" receptors (Fig. 6-1). Furthermore, there were no significant changes in the binding of ALDO-Type I receptor complexes to DEAE or GF/C filters prior and subsequent to activation (data not shown).

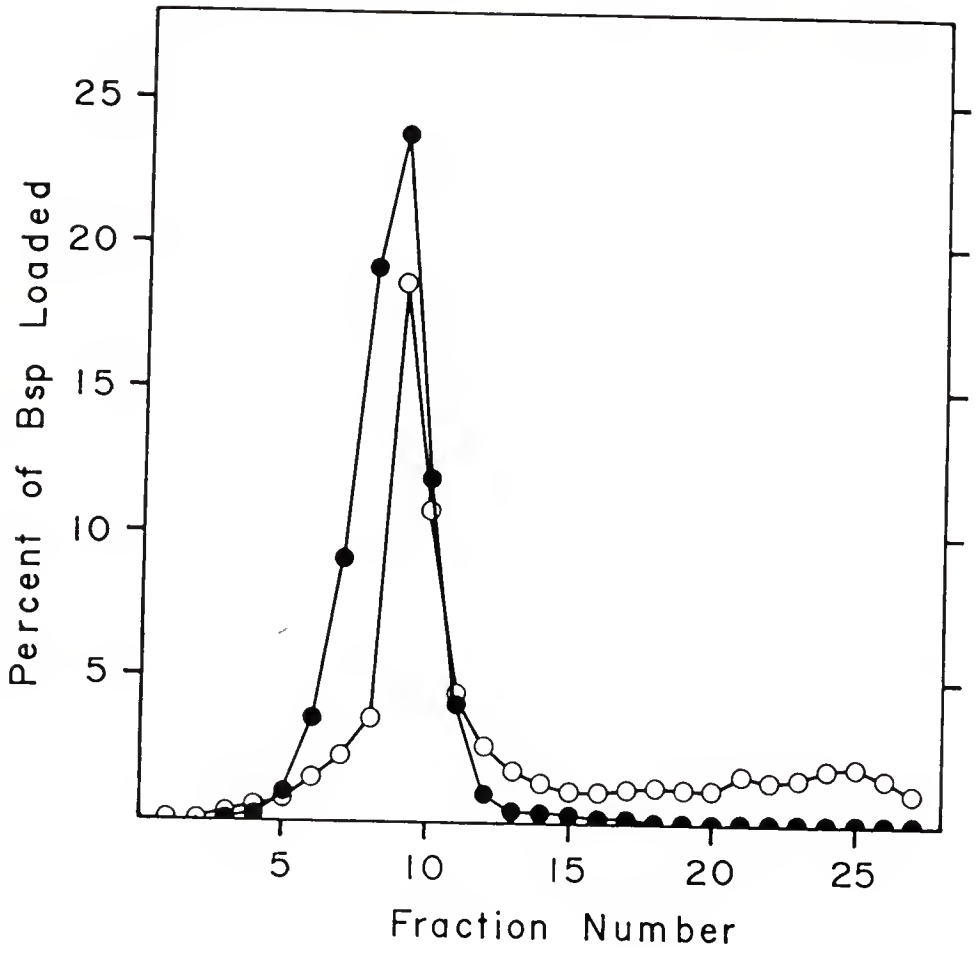
Table 6-2. Cytosol prepared in Buffer M_{20} was incubated with 10 nM [^{3}H]ALDO and 5 uM RU 26988 and either with (B_{NS}) or without (B_T) 2 uM [^{1}H]ALDO for 24 h at 0°C. Following this incubation period, 0.5 ml fractions were loaded on Sephadex G-25 columns (pre-equilibrated with Buffer K) to remove free steroids and molybdate and to bring the cytosol to the composition of Buffer K. Subsequently, sufficient 100 mM phenylmethyl sulfonylfluoride (PMSF) dissolved in absolute ethanol was added to cytosol to yield a final 1 mM concentration of this compound. Activation was performed as described under "Materials and Methods." DNA-C binding assays were performed in the presence of Buffer A or Buffer M_5 as described under "Materials and Methods."

Table 6-2

Effects of 5 mM Molybdate on the Binding of ALDO-Type I Receptor Complexes to DNA-Cellulose Before and After Salt- and Temperature-Induced Activation

BUFFER	TEMPERATURE	
	0°C	22°C
A	13 ± 2	14 ± 2
M_5	6 ± 1	20 ± 1

Figure 6-1. Comparison of the Surface Hydrophobic Properties of Unactivated and Activated ALDO-Type I Receptor Complexes on Pentyl-Agarose Columns. Cytosol prepared in Buffer DM_{20} was incubated with 10 nM [^{3}H]ALDO plus 5 uM RU 26988 and either with (B_{NS}) or without (B_{T}) 2 uM [^{1}H]ALDO for 24 h at 0°C. Following this incubation period, 0.5 ml fractions were loaded on Sephadex G-25 columns (pre-equilibrated with Buffer DM_{20} or Buffer DK) to remove free steroids (and molybdate under Buffer DK conditions) and to bring the cytosol to the composition of the desired buffers (i.e., DM_{20} or DK). Subsequently, sufficient 100 mM phenylmethyl sulfonylfluoride (PMSF) dissolved in absolute ethanol was added to cytosol to yield 1 mM concentration of this compound. Activation was then performed as described under "Materials and Methods." Unactivated groups underwent the same procedural steps except for the 22°C incabation. Pentyl-Agarose Hydrophobic interaction chromatography was performed as described in Chapter II. The data presented are expressed as a percent of total B_{SP} loaded on the columns and are from 3 independent replicate experiments.


Percent of Bsp Loaded
25
20
15
10
5
5
10
15
20
25
Fraction Number

Discussion

According to the two-step model of steroid hormone action (see Chapter I), a necessary step for modulation of DNA transcription is transformation (activation) of the receptors to a state with an increased affinity for DNA. The most commonly employed strategy for *in vitro* activation of steroid receptors is by increasing cytosolic salt concentration and/or temperature. Although this method has been employed successfully for Type II and other steroid receptors, the experiments described above, as well as two other recent reports (Eisen and Harmon, 1986; Schulman et al., 1986), suggest distinct differences between activation requirements of Type I and Type II receptors. In these studies, a complete activation of Type I receptors (measured by DEAE-cellulose column chromatography) could not be achieved: salt- and temperature-treatment of cytosol gave two distinct peaks of radioactivity one of which eluted at identical salt concentration as that of unactivated [^{3}H]ALDO-Type I receptor complexes.

Although earlier biochemical studies reported that nuclear uptake of ALDO-Type I receptors is a temperature-dependent process, autoradiographic techniques provide evidence for nuclear translocation to be temperature-independent (Bonvalet et al., 1984; Farman et al., 1984). It is suggested that although ALDO-receptor complex formation may occur in cytoplasm, the apparent temperature-dependency of nuclear "translocation" may be an artifact of biochemical techniques (Bonvalet et al., 1984; Farman et al., 1984). Recently, evidence from both biochemical, immunocytochemical and morphological studies have challenged the classical two-step model of steroid hormone action. The notion that unoccupied, unactivated steroid receptors may be localized

in the nucleus and that cytoplasmic localization may be an artifact of preparations has been implied for Type I, Type II and sex steroid receptor systems (e.g, Martin and Sheridan, 1982; Welshons et al., 1984 & 1985; Jensen et al., 1986; Pearce et al., 1986; for review see Walters, 1985; Gasc and Baulieu, 1986).

In light of these recent findings, and several other chemical differences between Type I and Type II receptors (Emadian et al., 1986) one may argue that Type I receptors do not undergo the same transformation process in order to modulate DNA transcription. In other words, it is entirely possible that unoccupied Type I receptors are associated with NM in a nonspecific manner. Upon binding ALDO, the receptors may then undergo some conformational changes that allow exposure of specific DNA-binding domains on the ligand-receptor complex, subsequent specific DNA-binding and hence modulation of mRNA synthesis. Alternatively, cytosol may contain some molybdate-like factors that act on Type I receptors and prevents the activation of the receptors (note that small molecular weight inhibitors of activation of Type II and other steroid receptors has been described previously). This latter possibility appears more likely since the results discussed in Chapter IV suggest the presence of such factor that inhibits optimal ligand binding capacity of Type I receptors.

It is also noteworthy that 300 mM KCl shown to activate Type II receptors (Table 6-1) was also found to inactivate unoccupied Type II receptors (see Chapter V). Because: 1) KCl and other agents capable of activating steroid hormone receptors do so by dissociating the monomeric steroid-binding subunit (92 kDa subunit) from the multimeric unactivated receptors (297 kDa oligomeric form) [i.e., from heat shock proteins

(90 kDa subunit), RNAs, and possibly other molecules]; 2) following ligand dissociation, activated Type II receptors appear to be unable to rebind ligand. If it is assumed that the inactivating mode of action of KCl of unoccupied Type II receptors is through subunit dissociation (Chapter V); since KCl alone does not activate occupied (Table 6-1) and inactivate unoccupied (Chapter V) Type I receptors, one may conclude that the difficulty in activating Type I receptors resides in the fact that subunit dissociation cannot occur because of the presence of molybdate-like factors in the cytosol (Chapter IV) (note that molybdate inhibits activation of steroid receptors by preventing subunit dissociation).

Future experiments will therefore focus on 1) procedures that may lead to full activation of Type I receptors (e.g., gel filtration of unlabeled cytosol in the absence of 2 mM molybdate followed by salt- and heat-induced activation); 2) characterization of possible endogenous inhibitors of activation of ALDO-Type I receptors; 3) tissue specificity of such factors and 4) physicochemical characterization of fully activated Type I receptors.

CONCLUDING REMARKS

Among the many original observations described in this dissertation, the most intriguing are the differences between Type I and Type II receptors. Note that these differences further extend the observations from an earlier study by our laboratory in which a number of chemical differences between Type I and Type II receptors were described (Emadian et al., 1986). Historically, the adrenocorticosteroid hormone receptors were classified in terms of their preference for a naturally occurring ligand. Therefore, on the basis their apparent affinity for ALDO and CORT, Type I and Type II receptors were classified as mineralo- and glucocorticoid receptors, respectively. The recent observations that CORT, in the absence of extravascular CBG, has an equal or greater affinity than ALDO for Type I receptors, has challenged the traditional classification of these receptors (for review see Funder et al., 1986; De Kloet et al., 1986). It is postulated that in CNS structures such as the hippocampus (where there is little or no extravascular CBG), the endogeous ligand for Type I receptors under basal conditions is CORT. Furthermore, under basal, but not "stress," conditions, Type II receptors are said to remain largely unoccupied (because of a 10-fold lower affinity of CORT for Type II than Type I receptors). Accordingly, it is proposed that Type I receptors mediate "tonic" influences of CORT on brain, whereas Type II receptors mediate the stress effects of CORT as well as CORT action on feedback regulatory mechanisms involving the hypothalamo-pituitary-adrenal axis (De Kloet et al., 1986). At variance with this simplistic theory, Dallman and

co-workers (Dallman, personal communication) have recently shown that during the late afternoon hours, when circulating levels of CORT are at the acme of their circadian fluctuation, CORT-Type II receptor interactions do indeed appear to mediate the feedback suppression of ACTH. However, during the morning, when circulating levels of CORT are at their nadir, CORT-Type I receptor interactions appear to be the principle mechanism by which glucocorticoid suppression of ACTH is mediated.

While recent studies have provided indirect evidence in support of the above theoretical relationships, a clear proof for the proposed role of CORT- versus ALDO-Type I receptor complexes is still lacking. For example, preliminary studies from our laboratory suggest that CORT administration to adrenalectomized mice down-regulates both Type I and Type II receptors in brain, whereas ALDO down-regulates only Type I receptors (Luttge and Rupp, unpublished results). Although at this point it is not clear whether this Type I receptor down-regulation is a direct effect of CORT-binding to Type I receptors or an indirect effect of CORT-binding to Type II receptors (possibly through the production of a protein which negatively modulates the gene for Type I receptor mRNA).

It is also noteworthy that although ligand binding is thought to be a prerequisite for initiation of the genomic actions of steroid hormones, this is by no means a sufficient step. In light of our demonstrations of the distinct chemical differences between Type I and Type II receptors, as well as the ease with which these receptors undergo the transformation(s) necessary to recognize nuclear acceptor site(s), one may postulate that ALDO-Type I versus CORT-Type I receptor complexes may be differentially prevented from eliciting a genomic

response by one or more regulatory mechanisms. Furthermore, this selective modulation of Type I receptor activation may be target specific. For example, Orti et al. (1986) reported no difficulties with activation of ALDO-Type I receptor complexes obtained from spinal cord. Experiments investigating such target and ligand specificities for Type I receptor activation, similarties and differences between their nuclear acceptor site(s), as well as the possible physiological and functional relevance of such differences are currently under way in our laboratory.

REFERENCES

Addison, T.: In: On the Constitutional and Local Effects of Diseases of the Suprarenal Capsules. Highley, London (1855).

Afifi, F.: Diminution of bile flow and of liver uptake and biliary excretion of bromosulphthalein by aldosterone. Tohoku J. Exp. Med. 135:139-147 (1981).

Afifi, F.; Peignoux, M. and Auclair, C.: Effect of chronic administration of aldosterone on bile secretion, on liver cytochrome P-450, and on red blood cell sodium content. Tohoku J. Exp. Med. 129:241-247 (1979).

Aguilera, G.: Differential effects of atrial natriuretic factor on angiotensin II- and adrenocorticotropin-stimulated aldosterone secretion. Endocrinology 120:299-304 (1987).

Alberts, B. and Herrick, G.: DNA-cellulose Chromatography. Meth. Enzymol. 21:198-271 (1971).

Alexis, M.N.; Stylianopoulos, F.; Kitraki, E. and Sekeris, G.E.: The distribution and properties of the glucocorticoid receptor from rat brain and pituitary. J. Biol. Chem. 258:4710-4714 (1983).

Ali, M. and Vedeckis, W.V.: The glucocorticoid receptor protein binds to transfer RNA. Science 235:467-470 (1987).

Al-Khouri, H. and Greenstein, B.D.: Role of corticosteroid-binding globulin in interaction of corticosterone with uterine and brain progesterone receptors. Nature Lond. 287:58-60 (1980).

Amaral, L.; Kobkul, L.; Samuels, A.J. and Werthamer, S.: Human liver nuclear transcortin. Biochim. Biophys. Acta 362:332-345 (1974).

Anderson, N.S. and Fanestil, D.D.: Corticoid receptors in rat brain: Evidence for an aldosterone receptor. Endocrinology 98:676-684 (1976).

Andreasen, P.A.: Changes in net charge of glucocorticoid receptors by activation, and evidence for a biphasic activation kinetic. Mol. Cell. Endocrinol. 28:563-586 (1982).

Aperia, A. and Larsson, L.: Induced development of proximal tubular NaKATPase, basolateral cell membranes and fluid reabsorption. Acta Physiol. Scand. 121:133-141 (1984).

Aperia, A.; Larsson, L. and Zetterstrom, R.: Hormonal induction of Na-K-ATPase in developing proximal tubular cells. Am. J. Physiol. 241:F356-F360 (1981).

Armanini, D.; Kuhnle, U.; Witzgall, H.; Tietze, U.; Saule, H.; Schroeter, C.; Strasser, T.; Butenandt, I.; Bidlingmaier, F. and Weber, P.C.: The determination of mineralocorticoid receptors in human mononuclear leukocytes from patients with mineralocorticoid excess: Physiological and pathological implications. Clin. Exp. Hypertension 8:781-785 (1986).

Arnemann, J.; Chalepakis, G.; Slater, E. and Beato, M.: Differential regulation of gene expression by glucocorticoids and progestins. J. Cell. Biochem. Suppl. 11A: 93 (1987).

Back, F.J.; Oakenfull, D. and Smith, M.B.: Increased thermal stability of proteins in the presence of sugars. Biochemistry 18:5191-5196 (1979).

Bailly, A.; Sallas, N. and Milgrom, E.: A low molecular weight inhibitor of steroid receptor activation. J. Biol. Chem. 252:858-863 (1977).

Bailly, A.; Sovouret, J.-F.; Sallas, N. and Milgrom, E.: Factors modifying the equilibrium between activated and non-activated forms of steroid-receptor complexes. Eur. J. Biochem. 88:623-632 (1978).

Ballard, P.L.; Baxter, J.D.; Higgins, S.G.; Rousseau, G.G. and Tomkins, G.M.: General presence of glucocorticoid receptors in mammalian tissues. Endocrinology 94:998-1002 (1974).

Barger, A.C.; Berlin, R.D. and Tulenko, J.F.: Infusion of aldosterone, 9-α-fluoro-hydrocortisone and antidiuretic hormone into the renal artery of normal and adrenalectomized, unanesthetized dogs: Effect on electrolyte and water excretion. Endocrinology 62:804-815 (1958).

Barnett, C.A.; Schmidt, T.J. and Litwack, G.: Effects of calf intestinal alkaline phosphatase, phosphatase inhibitors, and phosphorylated compounds on the rate of activation of glucocorticoid-receptor complexes. Biochemistry 19:5446-5455 (1980).

Barrack, E.R.: The nuclear matrix of the prostate contains acceptor sites for androgen receptors. Endocrinology 113:430-432 (1983).

Barrack, E.R. and Coffey, D.S.: The specific binding of estrogens and androgens to the nuclear matrix of sex hormone responsive tissues. J. Biol. Chem. 255:7265-7275 (1980).

Barrack, E.R. and Coffey, D.S.: Biological properties of the nuclear matrix: Steroid hormone binding. Rec. Prog. Hormone Res. 38:133-195 (1982).

Bastl, C.P.; Barnett, C.A.; Schmidt, T.J. and Litwack, G.: Glucocorticoid stimulation of sodium absorption in colon epithelia is mediated by corticosteroid IB receptor. J. Biol. Chem. 259:1186-1195 (1984).

Beato, M.; Kalimi, M.; Beato, W. and Feigelson, P.: Interaction of glucocorticoids with rat liver nuclei. Effect of adrenalectomy and cortisol administration. Endocrinology 94:377-387 (1974).

Beaumont, K.: Rat C6 glioma cells contain type I as well as type II corticosteroid receptors. Brain Res. 342:252-258 (1985).

Beaumont, K. and Fanestil, D.D.: Characterization of rat brain aldosterone receptors reveals high affinity for corticosterone. Endocrinology 113:2043-2051 (1983).

Beck, J.S. and Goren, H.J.: Stimulation of association curves and 'Scatchard' plots of binding reactions where ligand and receptor are degraded or internalized. J. Receptor Res. 3:561-577 (1983).

Bell, P.A. and Munck A.: Steroid-binding proteins and stabilization of cytoplasmic glucocorticoid receptors. Biochem. J. 136:97-107 (1973).

Berecek, K.H. and Bohr, D.F.: Structural and functional changes in vascular resistance and reactivity in the deoxycorticosterone acetate (DOCA)-hypertensive pig. Circ. Res. 40 (Suppl.I): I-146-152 (1977).

Berecek, K.H. and Bohr, D.F.: Whole body vascular reactivity during the development of deoxycorticosterone acetate hypertension in the pig. Circ. Res. 42:764-771 (1978).

Berezney, R. and Buckholtz, L.A.: Dynamic association of replicating DNA fragments with the nuclear matrix of regenerating liver. Exp. Cell Res. 132:1-13 (1981).

Bia, M.J.; Tyler, K. and DeFonzo, R.A.: The effect of dexamethasone on renal electrolyte excretion in the adrenalectomized rat. Endocrinology 111:882-888 (1982).

Birmingham, M.K.; Kraulis, I.; Traikov, H.; Bartova, A.; Li, M.P.; Chan, T.H.; Oliver, J.T. and Passanza, G.: Biological consequences of 18-hydroxylation. J. Steroid Biochem. 5:789-794 (1974).

Birmingham, M.K.; Sar, M. and Stumpf, W.E.: Localization of aldosterone and corticosterone in the central nervous system, assessed by quantitative autoradiography. Neurochem. Res. 9:333-350 (1984).

Blanchardie, P.; Lustenberger, P.; Orsonneau, J.L.; Denis, M. and Bernard, S.: Influence of molybdate, ionic strength and pH on ligand binding to the glucocorticoid receptor. Steroids 44:159-174 (1984).

Blazer-Yost, B.; Geheb, M.; Preston, A.; Handler, J. and Cox, M.: Aldosterone-induced proteins in renal epithelia. Biochim. Biophys. Acta 719:158-161 (1982).

Bodwell, J.E.; Holbrook, N.J. and Munck, A.: Sulfhydryl-modifying reagents reversibly inhibit binding of glucocorticoid-receptor complexes to DNA-cellulose. Biochemistry 23:1392-1398 (1984).

Bonvalet, J.P.; Manillier, C. and Farman, N.: Autoradiographic study of thermo-dependent nucleo-cytoplasmic distribution of aldosterone binding sites in intact target cells. J. Steroid Biochem. 20:325-328 (1984).

Brandts, J.F. and Hunt, L.: The thermodynamics of protein denaturation. III. The denaturation of ribonuclease in water and in aqueous urea and aqueous ethanol mixtures. J. Am. Chem. Soc. 89:4826-4838 (1967).

Brocklehurst, K.: Specific covalent modification of thiols: Application in the study of enzymes and other biomolecules. Intl. J. Biochem. 10:259-274 (1979).

Bruchovsky, N.; Rennie, P.S. and Comeau, T.: Partial purification of nuclear androgen receptor by micrococcal nuclease digestion of chromatin and hydrophobic interaction chromatography. Eur. J. Biochem. 120:399-405 (1981).

Bukhari, A.R.; Hartman, H.G. and Jouan, P.: Induction of the synthesis of thymidine kinase by aldosterone in the kidney of the immature male rat. Experentia 41:1449-1451 (1985).

Buttyan, R.; Olson, C.A.; Sheard, B. and Kallos, J.: Steroid receptor-nuclear matrix interactions. The role of DNA. J. Biol. Chem. 258:14366-14370 (1983).

Cake, M.H.; Goidl, A.J.; Parchman, L.G. and Litwack, G.: Involvement of a low molecular weight component(s) in the mechanism of the action of glucocorticoid receptor. Biochem. Biophys. Res. Commun. 71:45-52 (1976).

Cake, M.H. and Litwack, G.: The glucocorticoid receptor. In: Biochemical Actions of Hormones. (G. Litwack, Ed.). Vol. 3 pp. 317-390, Academic Press, New York, NY (1975).

Campbell, D.J.; Mendelsohn, F.A.O.; Adam, W.R. and Funder, J.W.: Is aldosterone secretion under dopaminergic control? Cir. Res. 49:1217-1227 (1981).

Campen, T.J.; Vaughn, D.A. and Fanestil, D.D.: Mineralo- and glucocorticoid effects on renal excretion of electrolytes. Pflugers Arch. 399:93-101 (1983).

Capco, D.G.; Wan, K. M. and Penman, S: The nuclear matrix: Three dimensional architecture and protein composition. Cell 29:847-858 (1982).

Carey, R.M. and Drake, Jr. C.R.: Dopamine selectively inhibits aldosterone responses to angiotensin II in humans. Hypertension 8:399-406 (1986).

Carey, R.M. and Sen, S.: Recent progress in the control of aldosterone secretion. Rec. Prog. Horm. Res. 42:251-296 (1986).

Carstensen, H; Bergers, C.A.J. and Li, C.H.: Demonstration of aldosterone and corticosterone as the principal steroids formed in incubates of adrenals of the American bullfrog (Rana catesbeiana) and stimulation of their production by mammalian adrenocorticotropin. J. Comp. Endocrinol. 1:37-50 (1961).

Catt, K.J.; Harwood, J.P.; Aguilera, G. and Dufau, M.L.: Hormonal regulation of peptide receptors and target cell responses. Nature Lond. 280:109-116 (1979).

Chambon, P.; Berry, M.; Bocquel, M.T.; Devic, M.; Govindan, M.V.; Green, S.; Gronemeyer, H.; Jeltsch, J.M.; Krosowski, Z.; Krust, A.; Kumar, V.; Meyer, M.; Stock, G.; Stricker, C.; Stropp, U. and Turcotte, B.: Steroid hormone receptors: Structure-function relationships. J. Cell. Biochem. Suppl. 11A: 86 (1987).

Chaplin, E.R.; Free, R.G. and Goldstein, G.W.: Inhibition by steroids of the uptake of potassium by capillaries isolated from rat brain. Biochem. Pharmacol. 30:241-245 (1981).

Chartier, L.; Schiffrin, E. and Thibault, G.: Effect of atrial natriuretic factor (ANF)-related peptides on aldosterone secretion by adrenal glomerulosa cells: Critical role of the intramolecular disulphide bond. Biochem. Biophys. Res. Commun. 122:171-174 (1984).

Chen, P.S.; Mills, I.H. and Bartter, F.C.: Ultrafiltration studies of steroid-protein binding. J. Endocrinol. 23:129-137 (1961).

Chou, Y.-C. and Luttge, W.G.: Glucocorticoids do not rebind to activated Type II receptors: Relationship to progesterone's antiglucocorticoid actions. The 69th Annual Endocrine Society Meeting, Indianapolis, IN (1987).

Cidlowski, J.A. and Cidlowski, N.B.: Regulation of glucocorticoid receptors by glucocorticoids in cultured HeLa S3 cells. Endocrinology 109:1975-1982 (1981).

Ciejek, E.M.; Nordstrom, J.L.; Ming-Jer, T. and O'Malley, B.W.: Ribonucleic acid precursors are associated with the chick oviduct nuclear matrix. Biochemistry 21:4945-4953 (1982).

Civan, M.M. and Hoffman, R.E.: Effect of aldosterone on electrical resistance of toad bladder. Am. J. Phsiol. 220:324-328 (1971).

Claire, M.; Oblin, M.-E.; Steimer, J.-L.; Nakane, H.; Misumi, J.; Michaud, A. and Corvol, J.: Effect of adrenalectomy and aldosterone on the modulation of mineralocorticoid receptors in rat kidney. J. Biol. Chem. 256:142-147 (1981).

Claire, M.; Rafestin-Oblin, M.-E.; Michaud, A,; Corvol, P,; Venot, A.; Roth-Meyer, C.; Boisvieux, J.F. and Mallet, A.: Statistical test of models and computerized parameter estimation for aldosterone binding in rat kidney. FEBS Lett. 88:295-299 (1978).

Claire. M.; Rafestin-Oblin, M.-E.; Michaud, A.; Roth-Meyer, C. and Corvol, P.: Mechanism of action of a new antialdosterone compound, prorenone. Endocrinology 104:1194-1200 (1979).

Claire, M.; Steimer, J.-L.; Oblin, M.-E.; Gaeggeler, H.-P.; Venot, A.; Corvol, P. and Rossier, B.C.: Cytoplasmic and nuclear uptake of aldosterone in toad bladder: A mathematical modeling approach. Am. J. Physiol. 248:C88-C101 (1985).

Coirini, H.; Magarinos, A.; De Nicola, A.F.; Rainbow, T.C. and McEwen, B.S.: Further studies of brain aldosterone binding sites employing new mineralocorticoid and glucocorticoid receptor markers in vitro. Brain Res. 361:212-216 (1985).

Coirini, H.; Marusic, E.T.; De Nicola, A.F.; Rainbow, T.C. and McEwen, B.S.: Identification of mineralocorticoid binding sites in rat brain by competition studies and density gradient centrifugation. Neuroendocrinology 37:354-360 (1983).

Compton, J.G.; Schrader, W.T. and O'Malley, B.W.: Selective binding of chick progesterone receptor A subunit to a DNA fragment containing ovalbumin gene sequences. Biochem. Biophys. Res. Commun. 105:96-104 (1982).

Compton, J.G.; Schrader, W.T. and O'Malley, B.W.: DNA sequence preference of the progesterone receptor. Proc. Natl. Acad. Sci. U.S.A. 80:16-20 (1983).

Conn, J.W.: Primary aldosteronism: A new clinical syndrome. J. Lab. Clin. Med. 45:3-17 (1955).

Cortas, N.; Abras, E. and Walser, M.: Aldosterone response in the turtle bladder is associated with an increase in ATP. Am. J. Physiol. 245:F512-F514 (1983).

Crabbe, J.: Stimulation of active sodium transport across the isolated toad bladder after injection of aldosterone to the animal. Endocrinology 69:673-682 (1961).

Crabbe, J. and de Weer, P.: Action of aldosterone and vasopressin on active transport of sodium by the isolated toad bladder. J. Physiol. 180:560 (1965).

Creighton, T.E.: In: Proteins. pp. 293-295, W.H. Freeman Co. Ltd., New York, NY (1984).

Crow, L.T.: Subcommissural organ, lateral hypothalamus, and dorsal logitudinal fasciculus in water and salt metabolism. Anat. Rec. 157:457-464 (1967).

Dahmer, M.K.; Housley, P.R. and Pratt, W.B.: Effects of molybdate and endogenous inhibitors on steroid-receptor inactivation, transformation, and translocation. Ann. Rev. Physiol. 46:67-81 (1984).

Dahmer, M.K., Quasney, M.W.; Bissen, S.T. and Pratt, W.B.: Molybdate permits resolution of untransformed glucocorticoid receptors from the transformed state. J. Biol. Chem. 256:9401-9505 (1981).

Danielson, M.; Northop, J. and Ringold, G.M.: Mutational analysis of the glucocorticoid receptor. J. Cell. Biochem. Suppl. 11A:104 (1987).

Daughaday, W.H.; Holloszy, J. and Martiz, I.K.: Binding of corticosteroids by plasma proteins. VI. The binding of cortisol and aldosterone by corticosteroid-binding globulin and by the estrogen-induced binding system of plasma. J. Clin. Endocrinol. Met. 21:53-61 (1960).

Davidson, E.T.; DeVenuto, F. and Westphal, U.: Steroid-protein interactions: IX. Interaction of aldosterone with human serum proteins in competition with spirolactones and other steroids. Endocrinology 71:893-900 (1962).

Davis, J.O.: Regulation of aldosterone secretion. In: The Handbook of Physiology: Adrenal Physiology (H. Blascko, G. Sayers and A.D. Smith, Eds.), Section 7, Vol. 6, pp. 77-106, American Physiological Society, Washington, D.C., (1975).

Defay, R.; Astruc, M.; Beseme, F.; Descomps. B. and Crastes de Paulet, A.: Physico-chemical properties of the hydroxysterol binding protein of human lymphocyte cytosol. Effects of high salt concentration and molybdate. FEBS Lett. 173:319-326 (1984).

De Kloet, E.R.: Function of steroid receptor systems in the central nervous system. Clin. Neuropharmacol. 7:272-280 (1984).

De Kloet, E.R. and McEwen, B.S.: A putative glucocorticoid receptor and transcortin-like macromolecule in pituitary cytosol. Biochim. Biophys. Acta 421:115-123 (1976a).

De Kloet, E.R. and McEwen, B.S.: Differences between cytosol receptor complexes with corticosterone and dexamethasone in hippocampal tissue from rat brain. Biochim. Biophys. Acta 421:124-132 (1976b).

De Kloet, E.R.; Reul, J.M.H.M.; De Paul, F.S.W.; Bloemers, M. and Ratka, A.: Function and plasticity of brain corticosteroid receptor systems: action of neuropeptides. J. Steroid Biochem. 25:723-731 (1986).

De Kloet, E.R.; Veldhuis, H.D.; Wagenaars, J.L. and Bergink, E.W.: Relative binding affinity of steroids for the corticosterone receptor system in rat hippocampus. J. Steroid Biochem. 21:173-178 (1984).

De Kloet, E.R.; Versteeg, D.H.G. and Kovacs, G.L.: Aldosterone blocks the response to corticosterone in the raphe-hippocampal serotonin system. Brain Res. 264:323-327 (1983).

De Nicola, A.F.; Tornello, S.; Weisenberg, L.; Fridman, O. and Birmingham, M.K.: Uptake and binding of [^{3}H]-aldosterone by the anterior pituitary and brain regions in adrenalectomized rats. Hormone Met. Res. 13:103-106 (1981).

Denison, M.S.; Vella. L.M. and Okey, A.B.: Hepatic Ah receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin. Partial stabilization by molybdate. J. Biol. Chem. 261:10189-10195 (1986).

Densmore, C.L.: Mechanisms of glucocorticoid Type II receptor regulation and activation in brain. Ph.D. Dissertation. University of Florida (1986).

Densmore, C.L. and Luttge, W.G.: ATP-dependent stabilization against microsomal factor-induced loss of unoccupied glucocorticoid receptors. Biochem. Intl. 10:633-639 (1985).

Densmore, C.L.; Chou, Y.-C. and Luttge, W.G.: Chemical nature of a potentially down-regulated form of the glucocorticoid receptor in brain cytosol. Soc. Neurosci. Abstr. 12:615 (1986a).

Densmore, C.L.; Luttge, W.G. and Chou, Y.-C.: Glucocorticoid receptor activation leads to changes in surface hydrophobicity as determined by hydrophobic interaction chromatography. Endocrinology 118 (Suppl.):264 (1986b).

Densmore, C.L.; Luttge, W.G. and Emadian, S.M.: Effects of metal ions and chelating agents on in vitro stability of glucocorticoid receptors in brain cytosol. Life Sci. 35:2237-2246 (1984a).

Densmore, C.L.; Luttge, W.G. and Emadian, S.M.: In vitro investigations of the potential regulatory role of sulfhydryl groups in mouse brain glucocorticoid receptors. Soc. Neurosci. Abstr. 10:1132 (1984b).

Devenport, L.D.: Adrenal modulation of brain size in adult rats. Beh. Neural Biol. 27:218-221 (1979).

Devenport, L.D. and Devenport, J.A.: The effects of adrenal hormones on brain and body size. Physiol. Psychol. 10:339-404 (1983).

Devenport, L.D.; Torres, A. and Murray, C.G.: Effects of aldosterone and deoxycorticosterone on food intake and body weight. Beh. Neurosci. 97:667-669 (1983).

Dias, J.A.; Huston, J.S. and Reichert, Jr.L.E.: Effect of the structure-stabilizing agent glycerol on detergent-solubilized follicle-stimulating hormone receptors from calf testis. Endocrinology 109:736-742 (1981).

Distelhorst, C.W. and Benutto, B.M.: Activation of the rat liver cytosol glucocorticoid receptor by Sephacryl S-300 filtration in the presence and absence of molybdate. Physical properties of the receptor and evidence of an activation inhibitor. J. Biol. Chem. 260:2153-2159 (1985).

Doucet, A. and Katz, A.I.: Mineralocorticoid receptors along the nephron: [^{3}H]aldosterone binding in rabbit tubules. J. Am. Physiol. 241:F605-F611 (1981a).

Doucet, A. and Katz, A.I.: Short-term effect of aldosterone on Na-K-ATPase in single nephron segments. Am. J. Physiol. 241:F273-F278 (1981b).

Drouin, J.; Charron, J.; Jeannotte, L.; Nemer, M.; Plante, R.K. and Wrange, O.: The pro-opiomelanocortin gene: a model for negative regulation of transcription by glucocorticoids. J. Cell. Biochem. Suppl. 11A:95 (1987).

Dundore, R.L.; Wurpel, J.N.D.; Balaban, C.D.; Keil, L.C. and Severs, W.B.: Central effects of aldosterone infused into the rat subcommissural organ region. Neurosci. Res. 1:341-351 (1984).

Economidis, I.V. and Rousseau, G.G.: Association of the glucocorticoid hormone receptor with ribonucleic acid. FEBS Lett. 181:47-52 (1985).

Edelman, I.S.; Bogoroch, R. and Porter, G.A.: On the mechanism of action of aldosterone on sodium transport: The role of protein synthesis. Proc. Natl. Acad. Sci. U.S.A. 50:1169-1177 (1963).

Edelman, I.S. and Marver, D.: Mediating events in the action of aldosterone. J. Steroid Biochem. 12:219-224 (1980).

Eisen, L.P. and Harmon, J.M.: Activation of the rat kidney mineralocorticoid receptor. Endocrinology 119:1419-1426 (1986).

Eisen, L.P.; Harrison, R.W. and Harmon, J.M.: Immunochemical characterization of the rat kidney glucocorticoid receptor. The role of proteolysis on the formation of corticosteroid binder IB. J. Biol. Chem. 261:3725-3731 (1986).

El Mernissi, G. and Doucet, A.: Short-term effect of aldosterone on renal sodium transport and tubular Na-K-ATPase in the rat. Pflugers Arch. 399:139-146 (1983).

El Mernissi, G. and Doucet, A.: Specific activity of Na-K-ATPase after adrenalectomy and hormone replacement along the rabbit nephron. Pflugers Arch. 402:258-263 (1984).

Emadian, S.M. and Luttge, W.G.: Effects of molybdate on partially purified Type I receptors for [^{3}H]aldosterone in brain. Soc. Neurosci. Abstr. 12:614 (1986).

Emadian, S.M.; Luttge, W.G. and Densmore, C.L.: Chemical differentiation of Type I and Type II receptors for adrenal steroids in brain cytosol. J. Steroid Biochem. 24:953-961 (1986).

Epstein, A.N.: Mineralocorticoids and cerebral angiotensin may act together to produce sodium appetite. Peptides. 3:493-494 (1982).

Ermisch, A. and Ruhle, H.-J.: Autoradiographic demonstration of aldosterone-concentrating neuron populations in rat brain. Brain Res. 147:154-158 (1978).

Fanestil, D.D. and Kipnowski, J.: Molecular action of aldosterone. Klin. Wochen. 60:1180-1185 (1982).

Fanestil, D.D. and Park, C.S.: Steroid hormones and the kidney. Ann. Rev. Physiol. 43:637-649 (1981).

Farman, N. and Bonvalet, J.P.: Aldosterone binding in isolated tubules. III. Autoradiography along the rat nephron. Am. J. Physiol. 245:F606-F614 (1983).

Farman, N.; Kusch, M. and Edelman, I.S.: Aldosterone receptor occupancy and sodium transport in the urinary bladder of Bufo marinus. Am. J. Physiol. 235:C90-C96 (1978).

Farman, N.; Manillier, C. and Bonvalet, J.P.: Autoradiographic study of nuclear localization of aldosterone binding sites in intact renal cells: Lack of temperature dependency. J. Steroid. Biochem. 20:585-593 (1984).

Farman, N.; Vandewalle, A. and Bonvalet, J.P.: Aldosterone binding in isolated tubules. I. Biochemical determination in proximal and distal parts of the rabbit nephron. J. Am. Physiol. 242:F63-F68 (1982a).

Farman, N.; Vandewalle, A. and Bonvalet, J.P.: Aldosterone binding in isolated tubules. II. An autoradiographic study of concentration dependency in the rabbit nephron. J. Am. Physiol. 242:F69-F77 (1982b).

Farman, N.; Vandewalle, A. and Bonvalet, J.P.: Autoradiographic study of aldosterone and dexamethasone binding in isolated glomeruli of rabbit kidney. Am. J. Physiol. 243:F235-F242 (1982c).

Farman, N.; Vandewalle, A. and Bonvalet, J.P.: Binding of aldosterone to cytoplasmic and nuclear receptors of the rabbit kidney. Am. J. Physiol. 240:C20-C27 (1981).

Feil, P.D.; Glasser, S.R.; Toft, D.O. and O'Malley, B.W.: Progesterone binding in the mouse and rat uterus. Endocrinology 91:738-746 (1972).

Feldman, D.: Ontogeny of rat hepatic glucocorticoid receptors. Endocrinology 95:1219-1227 (1974).

Feldman, D.; Funder, J.W. and Edelman, I.S.: Evidence for a new class of corticosterone receptors in the rat kidney. Endocrinology 92:1429-1441 (1973).

Feldman, M.; Kallos, J. and Hollander, V.P.: RNA inhibits estrogen receptor binding to DNA. J. Biol. Chem. 256:1145-1148 (1981).

Fersht, A.: In: Enzyme Structure and Mechanism. 2nd ed., pp. 299-301, W.H. Freeman Co. Ltd., New York, NY (1985).

Field, M.J. and Giebisch, G.H.: Hormonal control of renal potassium excretion. Kidney Intl. 27:379-387 (1985).

Field, M.J.; Stanton, B.A. and Giebisch, G.H.: Differential acute effects of aldosterone, dexamethasone, and hyperkalemia on distal tubular potassium secretion in the rat kidney. J. Clin. Invest. 74:1792-1802 (1984).

Finch, L.; Haensler, G. and Thoenen, H.: Failure to induce experimental hypertension in rats after intraventricular injection of 6-hydroxydopamine. Br. J. Pharmacol. 44:356-357 (1972).

Fisher, K.A.; Welt, L.G. and Hayslett, J.P.: Dissociation of Na-K-ATP-ase specific activity and net reabsorption of sodium. Am J. Physiol. 228:1745-1749 (1975).

Fluharty, S.J. and Epstein, A.N.: Sodium appetite elicited by intracerebroventricular infusion of angiotensin II in the rat: II. Synergistic interaction with systemic mineralocorticoids. Behav. Neurosci. 97:746-758 (1983).

Fojo, A.T.; Reuben, P.M.; Whitney, P.L. and Awad, W.M., Jr.: Effect of glycerol on protein acetylation by acetic anhydride. Arch. Biochem. Biophys. 240:43-50 (1985).

Fraser, R.; Brown, J.J.; Lever, A.F.; Mason, P.A. and Robertson, J.I.S. Control of aldosterone secretion. Clin. Sci. Mol. Med. 56:389-399 (1979).

Fraser, R.; Brown, J.J.; Lever, A.F.; Morton, J.J. and Robertson, J.I.S.: The renin-angiotensin system and aldosterone in hypertension: A brief review of some aspects. Intl. J. Obesity 5 (Suppl. 1):115-123 (1981).

Fregly, M.J. and Luttge, W.G.: The adrenal cortex. In: Human Endocrinology: An Integrative Text. pp. 107-161, Elsevier, New York, NY, (1982).

Fregly, M.J. and Rowland, N.E.: Role of renin-angiotensin-aldosterone system in NaCl appetite of rats. Am. J. Physiol. 248:R1-R11 (1985).

Fregly, M.J. and Waters, I.W.: Effects of mineralocorticoids on spontaneous sodium chloride appetite of adrenalectomized rats. Physiol. Behav. 1:65-74 (1966).

Friedman, S.M. and Friedman, C.L.: Cell permeability, sodium transport, and the hypertensive process in the rat. Circ. Res. 39:433-41 (1976).

Friedman, S.M.; Nakashima, M. and Friedman, C.L.: Cell Na and K in the rat tail artery during the development of hypertension induced by deoxycorticosterone acetate. Proc. Soc. Exp. Biol. Med. 150:171-176 (1975).

Funder, J.W.: Adrenocorticoid receptors in the brain. In: Frontiers in Neuroendocrinology (W.F. Ganong and L. Martini, Eds.). Vol. 9 pp. 169-189, Raven Press, New York (1986).

Funder, J.W.; Feldman, D. and Edelman, I.S.: Specific aldosterone binding in rat kidney and parotid. J. Steroid Biochem. 3:209-218 (1972).

Funder, J.W.; Feldman, D. and Edelman, I.S.: Glucocorticoid receptors in rat kidney: The binding of tritiated-dexamethasone. Endocrinology 92:1005-1013 (1973a).

Funder, J.W.; Feldman, D. and Edelman, I.S.: The roles of plasma binding and receptor specificity in the mineralocorticoid action of aldosterone. Endocrinology 92:994-1004 (1973b).

Ganguly, M. and Westphal, U.: Steroid-protein interactions. XVII. Influence of solvent environment on interactions between human α_1-acid glycoprotein and progesterone. J. Biol. Chem. 243:6130-6139 (1968).

Ganong, W.F. and Mulrow, P.J.: Rate of change in sodium and potassium excretion after injection of aldosterone into aorta and renal artery of the dog. Am. J. Physiol. 195:337-342 (1958).

Garg, L.C.; Knepper, M.A. and Burg, M.B.: Mineralocorticoid effects on Na-K-ATPase in individual nephron segments. Am. J. Physiol. 240:F536-F544 (1981).

Garg, L.C.; Narang, N. and Wingo, C.S.: Glucocorticoid effects on Na-K-ATPase in rabbit nephron segments. Am. J. Physiol. 248:F487-F491 (1985).

Garty, H.: Mechanisms of aldosterone action in tight epithelia. J. Memb. Biol. 90:193-205 (1986)

Gasc, J.-M. and Baulieu, E.-M.: Steroid hormone receptors: Intracellular distribution. Biol. Cell 56:1-6 (1986).

Gavin, J.R.; Roth, J.; Neville, D.M., Jr.; Demeyts, P. and Buell, D.N.: Insulin dependent regulation of insulin receptor concentration: A direct demonstration in cell culture. Proc. Natl. Acad. Sci. U.S.A. 71:84-88 (1974).

Geering, K.; Claire, M.; Gaeggeler, H.-P. and Rossier, B.C.: Receptor occupancy vs. induction of Na^+-K^+-ATPase and Na^+ transport by aldosterone. Am. J. Physiol. 248:C102-C108 (1985).

Geering, K.; Girardet, M.; Born, C.; Kraehenbuhl, J.-P. and Rossier, B.C.: Hormonal regulation of (Na^+, K^+)-ATPase biosynthesis in the toad bladder. Effect of aldosterone and 3,5,3'-triiodo-l-thyronin. J. Biol. Chem. 257:10338-10343 (1982).

Geheb, M.; Alvis, R.; Hercker, E. and Cox, M.: Mineralocorticoid-specificity of aldosterone-induced protein synthesis in giant-toad (Bufo marinus) urinary bladders. Biochem. J. 214:29-35 (1983).

Geheb, M.; Alvis, R.; Owen, A.; Hercker, E. and Cox, M.: Steroid-induced protein synthesis in giant-toad (Bufo marinus) urinary bladders. Correlation with natriferic activity. Biochem. J. 218:221-228 (1984).

Geheb, M.; Huber, G.; Hercker, E. and Cox, M.: Aldosterone-induced proteins in toad urinary bladders. Identification and characterization using two-dimensional polyacrylamide gel electrophoresis. J. Biol. Chem. 256:11716-11723 (1981).

Gekko, K. and Timasheff, S.N.: Mechanism of protein stabilization by glycerol: Preferential hydration in glycerol-water mixtures. Biochemistry 20:4667-4676 (1981a).

Gekko, K. and Timasheff, S.N.: Thermodynamic and kinetic examination of protein stabilization by glycerol. Biochemistry 20:4677-4686 (1981b).

Gerlsma, S.Y.: Reversible denaturation of ribonuclease in aqueous solutions as influenced by polyhydric alcohols and other additives. J. Biol. Chem. 243:957-961 (1968).

Gerlsma, S.Y.: The effects of polyhydric and monohydric alcohols on the heat-induced reversible denaturation of chymotrypsinogen A. Eur. J. Biochem. 243:957-961 (1970).

Gerlsma, S.Y. and Stuur, E.R.: The effect of polyhydric and monohydric alcohols on the heat-induced reversible denaturation of lysozyme and ribonuclease. Intl. J. Peptide Protein Res. 4:377-383 (1972).

Gerlsma, S.Y. and Stuur, E.R.: The effects of combining two different alcohols on the heat-induced reversible denaturation of ribonuclease. Intl. J. Peptide Protein Res. 6:65-74 (1974).

Geschwendt, M. and Kittstein, W.: Transformation of the estrogen-receptor complex from chick oviduct in 2 steps. Mol. Cell. Endocrinol. 20:251-260 (1980).

Giannopoulos, G.: Ontogeny of glucocorticoid receptors in rat liver. J. Biol. Chem. 250:5847-5881 (1975).

Giannopoulos, G.: A comparative study of receptors for natural and synthetic glucocorticoid in fetal rabbit lung. J. Steroid Biochem. 7:553-559 (1976).

Gilbert, G.J.: Renal effect of subcommissural extract. Neurology 13:43-55 (1963).

Girardet, M.; Geering, K.; Gaeggeler, H.-P. and Rossier, B.C.: Control of transepithelial Na^+ transport and Na-K-ATPase by oxytocin and aldosterone. Am. J. Physiol. 251:F662-F670 (1986).

Goidl, J.A.; Dolan, K.P.; Cake, M.H.; Parchman, L.G. and Litwack, G.: Reversible activation of the glucocorticoid receptor. Federation Proc. 35:1365a (1976).

Goldman, A.S. and Katsumata, M.: Murine glucocorticoid receptors: New evidence for a discrete receptor influenced by H-2. Arch. Biochem. Biophys. 249:316-325 (1986).

Gomez-Sanchez, C.E.: The role of steriods in human essential hypertension. Biochem. Pharmacol. 31:893-897 (1982).

Gomez-Sanchez, C.E. and Gomez-Sanchez, E.P.: RU-26988--a new tool for the study of the mineralocorticoid receptor. Endocrinology 113:1004-1009 (1983).

Gomez-Sanchez, E.P.: Intracerebroventricular infusion of aldosterone induces hypertension in rats. Endocrinology 118:819-823 (1986).

Goodman, D.B.: The role of lipid metabolism in the response of the toad urinary bladder to aldosterone. Ann. N.Y. Acad. Sci. 372:30-38 (1981).

Granberg, J.P. and Ballard, P.L.: The role of sulfhydryl groups in the binding of glucocorticoids by cytoplasmic receptors of lung and other mammalian tissues. Endocrinology 100:1160-1168 (1977).

Gray, H.E.: Mouse brain glucocorticoid receptors. Ph.D. Dissertation. University of Florida (1982).

Gray, H.E. and Luttge, W.G.: CBG-like molecules contribute to corticosterone binding in brain cytosol. Trans. Am. Soc. Neurochem. 13:236 (1982).

Gregory, M.C.; Duval, D. and Meyer, P.: Changes in cardiac and hepatic glucocorticoid receptors after adrenalectomy. Clin. Sci. Mol. Med. 51:487-493 (1976).

Grekin, R.J. and Sider, R.S.: Aldosterone receptor assay in rat kidney cytosol. J. Steroid Biochem. 13:835-837 (1980).

Grekin, R.J. and Sider, R.S.: Aldosterone receptor measurements during changes in dietary sodium. Endocrinology 108:109-112 (1981).

Grippo, J.F.; Holmgren, A. and Pratt, W.B.: Proof that the endogenous, heat-stable glucocorticoid receptor-activating factor is thioredoxin. J. Biol. Chem. 260:93-97 (1985).

Grippo, J.F.; Tienrungroj, W.; Dahmer, M.K.; Housley, P.R. and Pratt, W.B.: Evidence that the endogenous heat-stable glucocorticoid receptor-activating factor is thioredoxin. J. Biol. Chem. 258:13658-13664 (1983).

Grizzle, W.E. and Dunlap, N.E.: Aldosterone blocks adrenal compensatory hypertrophy in the rat. Am. J. Physiol. 246:E305-E310 (1984).

Gross, J.B.; Imai, M. and Kokko, J.P.: A functional comparison of the cortical collecting tubule and the distal convoluted tubule. J. Clin. Invest. 59:1284-1294 (1975).

Gross, J.B. and Kokko, J.P.: Effect of aldosterone and potassium sparing diuretics on electrical potential differences across the distal nephron. J. Clin. Invest. 59:82-89 (1977).

Grundy, H.M.; Simpson, S.A. and Tait, J.F.: Isolation of a highly active mineralocorticoid from beef adrenal extract. Nature Lond. 169:795-796 (1952).

Gueriguian, J.L.; Sawyer, M.E. and Pearlman, W.H.: A comparative study of progesterone- and cortisol-binding activity in the uterus and serum of pregnant and non-pregnant women. J. Endocrinol. 61:331-345 (1974).

Haack, D.; Mohring, J.; Mohring, B.; Petri, M. and Hachenthal, E.: Comparative study on development of corticosterone and DOCA hypertension in rats. Am. J. Physiol. 233:F403-F411 (1977).

Hampl, R.; Starka, L.; Obenberger, J. and Rozsival, P.: Content and binding of aldosterone in human ocular lens. Endocr. Exper. 18:59-64 (1984).

Hampl, R.; Starka, L. and Obenberger, J.: Aldosterone binding in rabbit lens epithelium. Exp. Eye Res. 33:581-583 (1981).

Hansen, T.R. and Bohr, D.F.: Hypertension, transmural pressure and vascular smooth muscle response in rats. Circ. Res. 36:590-598 (1975).

Hierholzer, K. and Wiederholt, M.: Some aspects of distal tubular solute and water transport. Kidney Intl. 9:198-213 (1976).

Hierholzer, K.; Wiederholt, M.; Holzgrove, H.; Giebisch, G.; Klase, R.M. and Weindhager, E.E.: Micropuncture study of renal transtubular concentration gradient of sodium and potassium in adrenalectomized rats. Pflugers Arch. 385:193-210 (1965).

Higashihara, E.; Carter, N.W.; Pucacco, L. and Kokko, J.P.: Aldosterone effects on papillary collecting duct pH profile of the rat. J. Am. Physiol. 246:F725-F731 (1984).

Higgins, S.J.; Rousseau, G.G.; Baxter, J.D. and Tomkins, G.M.: Early events in glucocorticoid action: Activation of the steroid receptor and its subsequent specific nuclear binding in a cell-free system. J. Biol. Chem. 248:5866-5872 (1973).

Hinton, B.T. and Keefer, D.A.: Binding of [^{3}H]aldosterone to a single population of cells within the rat epididymis. J. Steriod Biochem. 23:231-233 (1985).

Hirsch, D.; Kashgarian, M.; Boulpaep, E.L. and Hayslett, J.P.: Role of aldosterone in the mechanism of adaptation on the initial collecting tubule. Kidney Intl. 26: 798-807 (1984).

Hirsch, D.; Pace, P.; Binder, H.J. and Hayslett, J.P.: Evidence that aldosterone influences transport in target tissues by dissimilar mechanisms. Am. J. Physiol. 248:F507-F512 (1985).

Horisberger, J.-D. and Diezi, J.: Effect of mineralocorticoids on Na^+ and K^+ excretion in the adrenalectomized rat. Am. J. Physiol. 245:F89-F99 (1983).

Horisberger, J.-D. and Diezi, J.: Inhibition of aldosterone-induced antinatriuresis and kaliuresis by actinomycin D. Am. J. Physiol. 246:F201-F204 (1984).

Housley, P.R.; Dahmer, M.K. and Pratt, W.B.: Inactivation of glucocorticoid-binding capacity by protein phosphatases in the presence of molybdate and complete reactivation by dithiothreitol. J. Biol. Chem. 257:8615-8618 (1982).

Housley, P.R.; Grippo, J.F., Dahmer M.K. and Pratt, W.B: Inactivation, activation, and stabilization of glucocorticoid receptors. In: Biochemical Actions of Hormones. Vol. XI pp. 347-376, Academic Press Inc., New York, NY (1984).

Housley, P.R. and Pratt, W.B.: Direct demonstration of glucocorticoid receptor phosphorylation by intact L-cells. J. Biol. Chem. 258:4630-4635 (1983).

Huang, D. and Cuatrecasas, P.: Insulin-induced reduction of membrane receptor concentrations in isolated fat cells and lymphocytes. J. Biol. Chem. 250:8251-8259 (1975).

Hubbard, J.R. and Kalimi, M.: Synergistic effect of molybdate plus dithiothreitol on stabilization, reactivation, and partial purification of the kidney glucocorticoid receptor. J. Biol. Chem. 257:14263-14270 (1982).

Hubbard, J.R. and Kalimi, M.: Inactivation of hepatic glucocorticoid receptors by heparin. Biochim. Biophys. Acta 755:363-368 (1983).

Hubbard, J.R. and Kalimi, M.: Influence of proteinase inhibitors on glucocorticoid receptor properties: Recent progress and future perspectives. Mol. Cell. Biochem. 66:101-109 (1985).

Hughes, M.R.; Compton, J.G.; Schrader, W.T. and O'Malley, B.W.: Interaction of the chick oviduct progesterone receptor with deoxyribonucleic acid. Biochemistry 20:2481-2491 (1981).

Ichii, S.: Depletion and replenishment of glucocorticoid receptor in cytosol of rat tissue after administration of various glucocorticoids. Endocrinol. Japon. 28:293-304 (1981).

Ishii, M.; Ymakado, M. and Murao, S.: The extrarenal effects of aldosterone on the distribution of extracellular fluid in conscious adrenalectomized-nephrectomized rats. Pflugers Arch. 404:273-277 (1985).

Ishohashi, F.; Terada, M.; Tsukanaka, K.; Nakanishi, Y. and Sakamoto, Y.: A low-molecular-weight translocation modulator and its interaction with a macromolecular translocation inhibitor of the activated receptor-glucocorticoid complex. J. Biochem. 88:775-781 (1980).

Jacobson, H.R.: Functional segmentation of the mammalian nephron. Am. J. Physiol. 241:F203-F218 (1981).

Jensen, E.V.; Suzuki, T.; Kawashima, T.; Stumpf, W.E.; Jungblut, P.W. and DeSombre, E.R.: A two-step mechanism for the interaction of estradiol with rat uterus. Biochemistry 59:632-638 (1986).

Johnson, J.R. and Green, S.W.: Aldosterone stimulates Na^+ transport without affecting citrate synthase activity in cultured cells. Biochim. Biophys. Acta 647:293-296 (1981).

Johnson, J.P.; Jones, D. and Weismann, W.P.: Hormonal regulation of Na^+-K^+-ATPase in cultured epithelial cells. Am. J. Physiol 251:C186-C190 (1986).

Jones, A.W. and Hart, R.G.: Altered ion transport in aortic smooth muscle during deoxycorticosterone acetate hypertension in the rat. Circ. Res. 37:333-341 (1975).

Jorkasky, D.; Cox, M. and Feldman, G.M.: Differential effects of corticosteriods on Na^+ transport in rat distal colon in vitro. Am. J. Physiol. 248:G424-G431 (1985).

Kalimi, M.; Colman, P. and Feigelson, P.: The "activated" hepatic glucocorticoid-receptor complex. Its generation and properties. J. Biol. Chem. 250:1080-1086 (1975).

Kalimi, M.; Beato, M. and Feigelson, P.: Interaction of glucocorticoids with rat liver nuclei. I. Role of the cytosol proteins. Biochemistry 12:3365-3371 (1983).

Kalimi, M. and Love, K.: Role of chemical reagents in the activation of rat hepatic glucocorticoid-receptor complex. J. Biol. Chem. 255:4687-4690 (1980).

Kallos, J.; Fasy, T. and Hollander, V.P.: Assessment of estrogen receptor-histon interactions. Proc. Natl. Acad. Sci. U.S.A. 78:2874-2878 (1981).

Kaplan, N.M.: The biosynthesis of adrenal steriods: effects of angiotensin II, adrenocorticotropin, and potassium. J. Clin. Invest. 44:2029-2039 (1965).

Karin, M.; Imagawa, M.; Chiu, R.; Bonder, M.; Dana, S.; Lefevre, C. and Imbra, R.: Regulation of transcription by steriod hormone receptors, tissue specific transacting factors and phorbol esters. J. Cell. Biochem. Suppl. 11A:93 (1987).

Katayama, S. and Yamaji, T.: A binding-protein for aldosterone in human plasma. J. Steroid Biochem. 16:185-192 (1982).

Katsumata, M.; Gupta, C. and Goldman, A.S.: Glucocorticoid receptor IB: Mediator of anti-inflammatory and teratogenic functions of both glucocorticoids and phenytoin. Arch. Biochem. Biophys. 243:385-395 (1985).

Katz, A.I.; Doucet, A. and Morel, F.: Na-K-ATPase activity along the rabbit, rat, and mouse nephron. Am. J. Physiol. 237:F114-F120 (1979).

Kaufmann, S.H.; Okret, S., Wikstrom, A.-C.; Gustafsson, J.-A. and Shaper, J.H.: Binding of the glucocorticoid receptor to the rat liver nuclear matrix. The role of disulfide bond formation. J. Biol. Chem. 261:11962-11967 (1986).

Kem, D.C.; Gomez-Sanchez, C.E.; Kramer, N.J.; Holland, O.B. and Higgins, J.R.: Plasma aldosterone and renin activity response to ACTH infusion in dexamethasone-suppressed normal and sodium-depleted man. J. Clin. Endocrinol. Met. 40:116-124 (1975).

Kenyon, C.J.; Saccoccio, N.A. and Morris, D.J.: Aldosterone effect on water and electrolyte metabolism. J. Endocrinol. 100:93-100 (1984).

Kim, C.: Nest building, general activity and salt preference of rats following hippocampal ablation. J. Comp. Physiol. Psych. 53:11-16 (1960).

Kinne, R. and Kirsten, R.: Der einflub von aldosterone auf die aktivitat mitochondrialer und cytoplasmaticher enzyme in der rattenniere. Pflugers Arch. 300:244-254 (1968).

Kinsella, J.L.; Freiberg, J.M. and Sachtor, B.: Glucocorticoid activation of Na^+/H^+ exchange in renal brush border vesicles: Kinetic effects. Am. J. Physiol. 248:F233-F239 (1985).

Kipnowski, J.; Park, C.S. and Fanestil, D.D.: Modification of carboxyl of Na^+ channel inhibits aldosterone action on Na^+ transport. Am. J. Physiol. 245:F726-F734 (1983).

Kirsh, T.M.; Miller-Diener, A. and Litwack, G.: The nuclear matrix is the site of glucocorticoid receptor complex action in the nucleus. Biochem. Biophys. Res. Commun. 137:640-648 (1986).

Kirsten, E.; Kirsten, R.; Leaf, A. and Sharp, G.W.G.: Increased activity of enzymes of the tricarboxylic acid cycle in response to aldosterone in the toad bladder. Pflugers Arch. 300:213-225 (1968).

Kirsten, E.; Kirsten, R. and Sharp, G.W.G.: Effects of sodium transport stimulating substances on enzyme activities in the toad bladder. Pflugers Arch. 316:26-35 (1970).

Kirsten, R. and Kirsten, E.: Redox state of pyridine nucleotides in renal response to aldosterone. Am. J. Physiol. 223:229-235 (1972).

Knox, F.G.; Burnett, J.C.; Kohan, Jr.D.E.; Spielman, W.S. and Strand, J.C.: Escape from the sodium retaining effects of mineralocorticoids. Kidney Intl. 17:263-276 (1980).

Koblinsky, M.; Beato, M.; Kalimi, M. and Feigelson, P.: Glucocorticoid binding proteins of rat liver cytosol. II. Physical characterization and properties of the binding proteins. J. Biol. Chem. 247:7879-7904 (1972).

Koch, B.; Lutz, B.; Briaud, B. and Mialhe, C.: Heterogeneity of pituitary glucocorticoid binding. Evidence for transcortin-like compound. Biochim. Biophys. Acta 444:497-507 (1976).

Kojima, I.; Kojima, K.; Shibata, H. and Ogata, E.: Mechanism of cholinergic stimulation of aldosterone secretion in bovine adrenal glomerulosa cells. Endocrinology 119:284-291 (1986).

Kolpakov, M.G.; Shulga, V.A.; Mertvetsov, N.P.; Selyatitskaya, V.G. and Salganik, R.I.: Interaction of aldosterone and corticosterone with cell nuclei of target organs under different functional states. J. Steroid Biochem. 5:917-923 (1974).

Korge, P. and Timpmann, S.: Binding constants and stability of dexamethasone-binding protein from rat heart. Exp. Clin. Endocrinol. 32:178-187 (1983).

Kornel, L.: Studies on the mechanism of mineralocorticoid-induced hypertension: Evidence for the presence of an in situ mechanism in the arterial wall for a direct action of mineralocorticoids. Clin. Biochem. 14:282-293 (1981).

Kornel, L.; Ramsay, C.; Kanamarlapudi, N.; Travers, T. and Packer, W.: Evidence for the presence in arterial walls of intracellular-molecular mechanism for the action of of mineralocorticoids. Clin. Exp. Hyper. A4:1561-1582 (1982a).

Kornel, L.; Kanamarlapudi, N.; Travers, T.; Taff, D.J.; Patel, N.; Chen, C.; Baum, R. and Raynor, W.J.: Studies on high affinity binding of mineralo- and glucocorticoids in rabbit aorta cytosol. J. Steroid Biochem. 16:245-264 (1982b).

Kotchen, T.A. and Guthrie, Jr.G.P.: Renin-angiotensin-aldosterone and hypertension. Endocr. Rev. 1:78-99 (1980).

Kraulis, I.; Foldes, G.: Traikov, H.; Bubrosky, B. and Birmingham, M.K.: Distribution, metabolism and biological activity of deoxycorticosterone in the central nervous system. Brain Res. 88:1-14 (1975).

Krozowski, Z.S. and Funder, J.W.: Mineralocorticoid receptors in the lung. Endocrinology 109:1811-1813 (1981).

Krozowski, Z.S. and Funder, J.W.: Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid specificity. Proc. Natl. Acad. Sci. U.S.A. 80:6056-6060 (1983).

Kusch, M.; Farman, N. and Edelman, I.S.: Binding of aldosterone to cytoplasmic and nuclear receptors of the urinary bladder epithelium of Bufo marinus. Am. J. Physiol. 235:C82-C89 (1978).

Lahav, M.; Dietz, T. and Edelman, I.S.: The action of aldosterone on sodium transport: Further studies with inhibitors of RNA and protein synthesis. Endocrinology 92:1685-1699 (1973).

Lan, N.C.; Karin, M.; Nguyen, T.; Weisz, A.; Birnbaum, M.J.; Eberhardt, N.L. and Baxter, J.D.: Mechanisms of glucocorticoid hormone action. J. Steroid Biochem. 20:77-88 (1984).

Lan, N.C.; Matulich, D.T.; Morris, J.A. and Baxter, J.D.: Mineralocorticoid receptor-like aldosterone-binding protein in cell culture. Endocrinology 109:1963-1970 (1981).

Lan, N.C.; Graham, B.; Baxter, F.C. and Baxter, J.D.: Binding of steroids to mineralocorticoid receptors: Implications for in vivo occupancy by glucocorticoids. J. Clin. Endocrinol. 54:332-342 (1982).

Latman, N.S.; Sorensen, J.R.J. and Kishore, V.: Effect of aldosterone on the serum zinc, copper, and magnesium concentrations in the rat. Hormone Res. 20:192-196 (1984).

Law, P.Y. and Edelman, I.S.: Effect of aldosterone on incorporation of amino acids into renal medullary proteins. J. Memb. Biol. 41:15-40 (1978a).

Law, P.Y. and Edelman, I.S.: Induction of citrate synthase by aldosterone in the rat kidney. J. Memb. Biol. 41:41-64 (1978b).

Leach, K.L.; Dahmer, M.K. and Pratt, W.B.: Glucocorticoid-receptor stabilization: relative effects of molybdate ion on inactivation by alkaline phosphatase and phospholipase A_2. J. Steroid Biochem. 18:105-107 (1983).

Leach, K.L.; Dahmer, M.K.; Hammond, N.D.; Sando, J.J. and Pratt, W.B.: Molybdate inhibition of glucocorticoid receptor inactivation and transformation. J. Biol. Chem. 254:11884-11890 (1979).

Leach, K.L.; Grippo, J.F.; Housley, P.R.; Dahmer, M.K.; Salive, M.E. and Pratt, W.B.: Characteristics of an endogenous glucocorticoid stabilizing factor. J. Biol. Chem. 257:381-388 (1982).

Lee, S.-M.K.; Chekal, M.A. and Katz, A.I.: Corticosterone binding sites along the nephron. Am. J. Physiol. 244:F504-F509 (1983).

Le Hir, M.; Kaissing, B. and Dubach, U.C.: Distal tubular segments of the rabbit kidney after adaptation to altered Na- and K-intake. II. Changes in Na-K-ATPase activity. Cell Tissue Res. 224:493-504 (1982).

Lehoux, J.-G.; Bellabarba, D. and Beaudry, C.: Corticosteroid receptors in the kidney of chick embryo. III. Nature, properties, and ontogeny of aldosterone receptor. Gen. Comp. Endocrinol. 53:116-125 (1984).

Liao, S.; Chang, C. and Saltzman, A.G.: Androgen-receptor interaction - an overview. In: Steroid Hormone Receptors. Structure and Function. (H. Eriksson and J.-A. Gustafsson, Eds.), pp. 407-418, Elsevier, New York, NY (1983).

Lim, R.C.; Nakayama, D.K.; Diglievi, E.G.; Schambelan, M. and Hunt, T.K.: Primary aldosteronism: Changing concepts in diagnosis and management. Am. J. Surgery. 152: 116-121 (1986).

Little, R.J.: Design and synthesis of several antiinflamatory soft steroids and analysis of factors involved in the biological activities thereof. Ph.D. Dissertation. University of Florida (1983).

Litwack, G.; Filler, R.; Rosenfield, S.A.; Lichtash, N.; Wishman, C.A. and Singer, S.: Liver cytosol corticosteroid binder II, a hormone receptor. J. Biol. Chem. 248:7481-7486 (1973).

Litwack, G. and Rosenfield, S.A.: Liver cytosol corticosteroid binder IB, a new binding protein. J. Biol. Chem. 250:6799-6805 (1975).

Livesey, J.H.; Roud, H.K.; Metcalf, M.G. and Donald, R.A.: Glycerol prevents loss of immunoreactive follicle-stimulating hormone and luteinizing hormone from frozen urine. J. Endocrinol. 98:381-384 (1983).

Llaurado, J.G.; Madden, J.A. and Smith, G.A.: Some effects of aldosterone on sodium kinetics and distribution in porcine arterial wall. Am. J. Physiol. 244:R553-R557 (1983).

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. and Randall, R.J.: Protein measurement with Folin phenol reagent. J. Biol. Chem. 193:265-275 (1951).

Lucas, G.H.W.: Blood and urine findings in desuprarenalized dogs Am. J. Physiol. 77:114-125 (1926).

Luttge, W.G.: Molecular mechanisms of steroid hormone actions. In: Hormones and Agressive Behavior. (B.B. Svare, Ed.) pp. 247-312, Plenum Press, New York, NY (1983).

Luttge, W.G. and Densmore, C.L.: Temperature-dependent kinetic correlates of the activation of the glucocorticoid-receptor complex. J. Neurochem. 42:242-247 (1984).

Luttge, W.G., Densmore, C.L. and Gray, H.E.: Kinetic analyses of activation-induced changes in the hydrodynamic and surface properties of the glucocorticoid-receptor complex in mouse brain. Neurochem. Res. 9:273-282 (1984a).

Luttge, W.G., Densmore, C.L. and Emadian, S.M.: Increased binding to DNA-cellulose of the unactivated and activated glucocorticoid-receptor complex from mouse brain following sucrose density gradient ultracentrifugation. Brain Res. 295:385-388 (1984b).

Luttge, W.G.; Emadian, S.M.; Densmore, C.L. and Slatick, R.S.: Efficacy of five methods of bound-free separation of gluco- and mineralocorticoids from Type I, II and III receptors found in HEPES- and TRIS-buffered mouse brain cytosol. Steroids 43:429-443 (1984c).

Luttge, W.G., Gray, H.E. and Densmore, C.L.: Hydrodynamic and biochemical correlates of the activation of the glucocorticoid-receptor complex. J. Steroid Biochem. 20:545-553 (1984d).

Magarinos, A.M.; Coirini, H.; De Nicola, A.F. and McEwen, B.S.: Mineralocorticoid regulation of salt uptake is preserved in hippocampectomized rats. Neuroendocrinology 44:494-497 (1986).

Markovic, R.D.; Eisen, H.J.; Parchman, L.G.; Barnett, C.A. and Litwack, G.: Evidence for a physical role of corticosteroid binder IB. Biochemistry 19:4556-4569 (1980).

Martin, P.M. and Scheridan, P.J.: Toward a new model for the mechanism of action of steroids. J. Steroid Biochem. 16:215-229 (1982).

Marver, D.: Aldosterone receptors in rabbit renal cortex and red medulla. Endocrinology 106:611-618 (1980).

Marver, D.: Models of aldosterone action on sodium transport: Emerging concepts. Adv. Exp. Med. Biol. 196:153-171 (1986).

Marver, D.; Goodman, D. and Edelman, I.S.: Relationships between renal cytoplasmic and nuclear aldosterone-receptors. Kidney Intl. 1:210-223 (1972).

Marver, D.; Stewart, J.; Funder, J.W.; Feldman, D. and Edelman, I.S.: Renal aldosterone receptors; Studies with [^{3}H]-aldosterone and the antimineralocorticoid [^{3}H]-spirolactone (SC-26304). Proc. Natl. Acad. Sci. U.S.A. 71:1431-1435 (1974).

Matsuoka, H.; Ishii, M.; Goto, A. and Sugimoto, T.: Role of serotonin type II receptors in regulation of aldosterone production. Am. J. Physiol. 249:E234-E238 (1985).

Mayer, M. and Litwack, G.: Corticosteroid binder IB: A model of glucocorticoid receptor diversity. J. Steroid Biochem. 19:323-326 (1983).

Mayer, M.; Schmidt, T.J.; Barnett, C.A.; Miller, A. and Litwack, G.: In vitro production of corticosteroid binder IB in the presence of proteolytic inhibitors. J. Steroid Biochem. 18:111-120 (1983).

Mayer, N.; Kaiser, N.; Micholland, N. and Rosen, F.: Cortisol binding in rat skeletal muscle. J. Biol. Chem. 250:1207-1211 (1975).

McBlain, W.A. and Shyamala, G.: Heparin-mediated inactivation and transformation of mammary cytoplasmic glucocorticoid receptor. J. Steroid Biochem. 12:219-224 (1980).

McBlain, W.A.; Toft, D.O. and Shyamala, G.: Transformation of mammary cytoplasmic glucocorticoid receptor under cell-free conditions. Biochemistry 20:6790-6798 (1981).

McDuffie, G.E., Jr.; Quinn, R.G. and Litovitz, T.A.: Dielectric properties of glycerol-water mixtures. J. Chem. Phys. 37:239-242 (1962).

McEwen, B.S.: Influence of adrenocortical hormone on pituitary and brain function. In: Glucocorticoid Hormone Action. (J.D. Baxter and G.G. Rousseau, Eds.) Vol. 12, pp. 467-492, Springer Publishing Co., Berlin, (1979).

McEwen, B.S.; De Kloet, E.R. and Rostene, W.: Adrenal steroid receptors and actions in the nervous system. Physiol. Rev. 66:1121-1188 (1986a).

McEwen, B.S.; Lambdin, L.T.; Rainbow, T.C. and De Nicola, A.F.: Aldosterone effects on salt appetite in adrenalectomized rats. Neuroendocrinology 43:38-43 (1986b).

McEwen, B.S.; Wallack, G. and Magnus, C.: Corticosterone binding to hippocampus: Immediate and delayed influences of the absence of adrenal secretion. Brain Res. 70:321-334 (1974).

McIntyre, W.R. and Samuels, H.H.: Triamcinolone acetonide regulates glucocorticoid-receptor levels by decreasing the half-life of the activated nuclear-receptor form. J. Biol. Chem. 260:418-427 (1985).

Meyer, C.J.; Layne, D.S.; Tait, J.F. and Pincus, G.: The binding of aldosterone to plasma proteins in normal, pregnant and steroid-treated women. J. Clin. Invest. 40:1663-1671 (1961).

Meyer, J.S.: Biochemical effects of corticosteroids on neural tissue. Physiol. Rev. 65:946-1020 (1985).

Miesfeld, R.; Godowski, P and Yamamoto, K.R.: Analysis of enhance-activation by the glucocorticoid receptor. J. Cell. Biochem. Suppl. 11A:88 (1987).

Milgrom, E. and Baulieu, E.-E.: Progesterone in uterus and plasma. I. Bindings in rat uterus 105,000 g supernatant. Endocrinology 87:276-287 (1970).

Milgrom, E.; Atger, M. and Baulieu, E.-E.: Acidophilic activation of steroid hormone receptors. Biochemistry 12:5198-5205 (1973a).

Milgrom, E.; Thi, L.; Atger, M. and Baulieu, E.-E.: Mechanisms regulating the concentration and the conformation of progesterone receptor(s) in the uterus. J. Biol. Chem. 248:6366-6374 (1973b).

Miller, L.K.; Tuazon, F.B.; Niu, E. and Sherman, M.R.: Human breast tumour estrogen receptor: Effects of molybdate and electrophoretic analysis. Endocrinology 108:1369-1378 (1981)

Miller-Dienen, A.; Schmidt, T.J. and Litwack, G.: Protein kinase activity associated with the purified rat hepatic glucocorticoid receptor. Proc. Natl. Acad. Sci. U.S.A. 82:4003-4007 (1985).

Moguilewsky, M. and Philibert, D.: RU 38486: Potent antiglucocorticoid activity correlated with strong binding to the cytosolic glucocorticoid receptor followed by an impaired activation. J. Steroid Biochem. 20:271-276 (1984).

Moguilewsky, M. and Raynaud, J.P.: Evidence for a specific mineralocorticoid receptor in rat pituitary and brain. J. Steroid Biochem. 12:309-314 (1980).

Monder, C.; Shackleton, C.H.L.; Bradlow, H.L.; New, M.I.; Stoner, E.; Iohan, F. and Lakshni, V.: The syndrome of apparent mineralocorticoid excess: Its association with 11β-dehydrogenase and 5α-reductase deficiency and some consequences for corticosteroid metabolism. J. Clin. Endocrinol. Met. 63:550-557 (1986).

Moudgil, V.K. and John, J.K.: ATP-dependent activation of glucocorticoid receptor from rat liver cytosol. Biochem. J. 190:799-808 (1980).

Moura, A.-M. and Worcel, M.: Direct action of aldosterone on transmembrane ^{22}Na efflux from arterial smooth muscle. Rapid and delayed effects. Hypertension 6:425-430 (1984).

Muldoon, T.G.: Regulation of steroid hormone receptor activity. Endocrine Rev. 1:339-364 (1980).

Muller, R.E.; Traish, A.M. and Wotiz, H.H.: Estrogen receptor activation precedes transformation. Effects of ionic strength, temperature, and molybdate. J. Biol. Chem. 258:9227-9236 (1983).

Mulvihill, E.R.; LePennee, J.-P. and Chambon, P.: Chicken oviduct progesterone receptor: Location of specific regions of high-affinity binding in cloned DNA fragments of hormone-responsive genes. Cell 28:621-632 (1982).

Munck, A. and Guyre, P.M.: Glucocorticoid physiology, pharmacology and stress. Adv. Exp. Med. Biol. 196:81-96 (1986).

Munck, A.; Guyre, P.M. and Holbrook, N.J.: Physiological functions of glucocorticoids in stress and their regulation of pharmacological actions. Endocrine Rev. 5:25-44 (1984).

Munck, A.; Wira, C.; Young, D.A.; Mosher, K.M.; Hallahan, C. and Bell, P.A.: Glucocorticoid-receptor complexes and the earliest steps in the action of glucocorticoids on thymus cells. J. Steroid Biochem. 3:567-578 (1972).

Murakami, N.; Quattrociocchi, T.M., Szocik, J.F. and Moudgil, V.K.: pH-dependent effects of sodium tungstate on the steroid-binding properties of hen oviduct progesterone receptor. Biochim. Biophys. Acta 719:267-272 (1982).

Muramatsu, M.; Tanaka, M.; Otomo, S. and Aihara, H.: Characteristics of glucocorticoid-binding sites of rat liver: Different effects of adrenalectomy on the binding. Japan. J. Pharmacol. 33:955-963 (1983).

Nakada, M.; Simpson, R.U. and DeLuca, H.F.: Molybdate and the 1,25-dihydroxyvitamin D_3 receptor from chick intestine. Arch. Biochem. Biophys. 238:517-521 (1985).

Naruse, M.; Obana, K.; Naruse, K.; Yamaguchi, H.; Demure, H.; Inagami, T. and Shizume, K.: Atrial natriuretic polypeptide inhibits cortisol secretion as well as aldosterone secretion in vitro from human adrenal tissue. J. Clin. Endocrinol. Met. 64:10-16 (1987).

Nelson, W.G.; Pienta, K.J.; Barrack, E.R. and Coffey, D.S.: The role of nuclear matrix in the organization and function of DNA. Ann. Rev. Biophys. Chem. 15:457-475 (1986).

Nicholls, M.G.; Ramsay, L.E.; Boddy, E; Fraser, R.; Morton, J.J. and Robertson, J.I.S.: Mineralocorticoid-induced blood pressure, electrolyte and hormone changes and reversal with spironolactone in healthy man. Metabolism 28:584-593 (1979).

Nielsen, C.J.; Sando, J.J. and Pratt, W.B.: Evidence that dephosphorylation inactivates glucocorticoid receptors. Proc. Natl. Acad. Sci. U.S.A. 74:1398-1402 (1977a).

Nielsen, C.J.; Sando, J.J.; Vogel, W.M. and Pratt, W.B.: Glucocorticoid receptor inactivation under cell-free conditions. J. Biol. Chem. 252:7568-7578 (1977b).

Nielsen, C.J.; Vogel, W.M. and Pratt, W.B.: Inactivation of glucocorticoid receptors in cell-free preparations of rat liver. Cancer Res. 37:3420-3426 (1977c).

Nishigori, H. and Toft, D.: Inhibition of progesterone receptor activation by sodium molybdate. Biochemistry 19:77-83 (1980).

Noma, K.; Nakao, K.; Sato, B.; Nishizawa, Y.; Matsumoto, K. and Yamamura, Y.: Effect of molybdate on activation and stabilization of steroid receptors. Endocrinology 107:1205-1211 (1980).

Nowaczynski, M.; Nowaczynski, W.; Mavoungou, D.; Lioy, F.; Wilkins, G.; Fung, K. and Boyko, W.: Aldosterone-binding globulin-induced hypertension in the rat. A new experimental model. Hypertension 5 (Suppl. V):V163-V171 (1983).

Oakenfull, D.G. and Fenwick, D.E.: Hydrophobic interaction in aqueous organic mixed solvents. J. Chem. Soc., Faraday Trans. 1 75:636-645 (1979).

Ogle, T.F.: Characterization of high affinity progesterone binding to rat placental cytosol. Endocrinology 106:1861-1868 (1980).

Ogle, T.F.: Action of glycerol and sodium molybdate in stabilization of the progesterone receptor from rat trophoblast. J. Biol. Chem. 258:4982-4988 (1983).

Okret, S.; Poellinger, L.; Dong, Y. and Gustafsson, J.A.: Down-regulation of glucocorticoid receptor m-RNA by glucocorticoid hormones and recognition by the receptor of a specific binding sequence with a receptor cDNA clone. Proc. Natl. Acad. Sci. U.S.A. 83:5899-5903 (1986).

Olefsky, J.M.: The insulin receptor: Its role in insulin resistance of obesity and diabetes. Diabetes 25:1154-1162 (1976).

O'Neil, R.G. and Dubinsky, W.P.: Micromethodology for measuring ATPase activity in renal tubules: Mineralocorticoid influence. Am. J. Physiol. 247:C314-C320 (1984).

O'Neil, R.G. and Hayhurst, R.A.: Sodium-dependent modulation of the renal Na-K-ATPase: Influence of mineralocorticoids on the cortical collecting duct. J. Memb. Biol. 85:169-179 (1985).

O'Neil, R.G. and Helman, S.I.: Transport characteristics of renal collecting tubules: Influence of DOCA and diet. Am. J. Physiol. 233:F544-F558 (1977).

Onoyama, K.; Bravo, E.L. and Tarazi, R.D.: Sodium, extracellular fluid volume, and cardiac output changes in the genesis of mineralocorticoid hypertension in the intact dog. Hypertension 1:331-336 (1979).

Orti, E.; Magarinos, A.M. and De Nicola, A.F.: Evidence of high affinity, stereoselective binding sites for [^{3}H]-Aldosterone in the spinal cord. Neuroendocrinology 43:404-409 (1986).

Osore, H. and Gilbert, J.: The action of aldosterone and ouabain on cation concentrations in incubated renal slices. Biochem. Pharm. 31:2571-2574 (1982).

Palem-Vliers, M.; Hacha, R.; Saint-Remy, A.; Fredericq, E. and Genard, P.: Physicochemical characterization of cytosol and nuclear mineralocorticoid receptors in rat kidney. J. Steroid Biochem. 16:457-461 (1982).

Palkovits, M.: Karyometrische untersuchungen zur klarung der osmo-bzw. volumen regularischen rolle des subcommissural organes und senier funktionellen verbindung mit der nebennierenrinde. Z. Zellforsch. 84:59-71 (1968).

Palkovits, M. and Folvari, P.I.: Effect of the subcommissural organ and the pineal body on the adrenal cortex. Endocrinology 72:28-32 (1963).

Palkovits, M.; Monos, E. and Fachet, J.: The effect of subcommissural organ lesions on aldosterone production in the rat. Acta Endocrinol. 48:169-176 (1965).

Palmer, L.G.; Li, J.H.-Y.; Lindemann, B. and Edelman, I.S.: Aldosterone control of the density of sodium channels in the toad urinary bladder. J. Memb. Biol. 64:91-102 (1982).

Palmer, L.G. and Speez, N.: Stimulation of apical Na permeability and basolateral Na pump of toad urinary bladder by aldosterone. Am. J. Physiol. 250:F273-F281 (1986).

Park, C.S. and Edelman, I.S.: Effect of aldosterone on abundance and phosphorylation kinetics of Na-K-ATPase of toad urinary bladder. Am. J. Physiol. 246:F509-F516 (1984a).

Park, C.S. and Edelman, I.S.: Dual action of aldosterone on toad bladder: Na^+ permeability and Na^+ pump modulation. Am. J. Physiol. 246:F517-F525 (1984b).

Pearce, P.T.; Lipkevicius, O.R. and Funder, J.W.: High affinity (Type I) aldosterone-binding sites in rat epididymis. Endocrinology 118:2072-2075 (1986a).

Pearce, P.T.; McNally, M. and Funder, J.W.: Nuclear localization of Type I aldosterone binding sites in steroid-induced GH_3 cells. Clin. Exp. Pharmacol. Physiol. 13:647-654 (1986b).

Perrot-Applanat, M. and Milgrom, E.: Messenger ribonucleic acid for corticosteroid-binding globulin. Translation and preliminary characterization. Biochemistry 18:5732-5737 (1979).

Petty, K.J.; Kokko, J.P. and Marver, D.: Secondary effect of aldosterone on Na-K-ATPase activity in the rabbit cortical collecting tubule. J. Clin. Invest. 68:1514-1521 (1981).

Pfahl, M.: Specific binding of the glucocorticoid-receptor complex to the mouse mammary tumor proviral promotor region. Cell 31:475-482 (1982).

Potgieter, H.C.; Kline, T.; Magagane, F.; Savage, N. and Wittliff, J.L.: Molybdate and the molecular properties of the vervet monkey estrogen receptor. J. Steroid Biochem. 25:951-956 (1986).

Pratt, W.B. and Ishii, D.N.: Specific binding of glucocorticoids in vitro in the soluble fraction of mouse fibroblasts. Biochemistry 11:1401-1410 (1972).

Puca, G.A.; Nola, E.; Sica, V. and Bresciani, F.: Estrogen-binding proteins of calf uterus. Partial purification and preliminary characterization of two cytoplasmic proteins. Biochemistry 10:3769-3779 (1971).

Quirk, S.J.; Gannell, J.E. and Funder, J.W.: Aldosterone-binding sites in pregnant and lactating rat mammary glands. Endocrinology 113:1812-1817 (1983).

Raaka, B.M. and Samuels, H.H.: Regulation of thyroid hormone nuclear receptor levels in GH_1 cells by 3,5,3'-triiodo-l-thyronin. Use of dense amino acid labeling to determine the influence of hormone on the receptor half-life and the rate of appearance of newly synthesized receptor. J. Biol. Chem. 256:6883-6889 (1981).

Raaka, B.M. and Samuels, H.H.: The glucocorticoid receptor in GH_1 cells. Evidence from dense amino acid labeling and whole cell studies for an equilibrium model explaining the influence of hormone on the intracellular distribution of receptor. J. Biol. Chem. 258:417-425 (1983).

Rafestin-Oblin, M.-E.; Michaud, A.; Claire, M. and Corvol, P.: Dramatic protective effect of ligand against thermal degradation on mineralo- and glucocorticoid receptors of rat kidney. J. Steroid Biochem. 8:19-23 (1977).

Rafestin-Oblin, M.-E.; Claire, M.; Lombes, M.; Michaud, A. and Corvol, P.: Modulation of aldosterone receptors in rat kidney: Effects of steroid treatment and potassium diet. J. Steroid Biochem. 21:465-470 (1984).

Ramaley, R.F. and Vasantha, N.: Glycerol protection and purification of Bacillus subtilis glucose dehydrogenase. J. Biol. Chem. 258:12558-12565 (1983).

Rarey, K.E. and Luttge, W.G.: Presence of Type I and Type II receptors for adrenocorticosteroid hormones in the inner ear. The 9th Mid-Winter Meeting for Assc. for Res. in Oto. Clearwater, FL (1987).

Raynaud, J.P.; Bouton, M.M.; Moguilewsky, M.; Ojasoo, T.; Philibert, D.; Beck, G.; Labrie, F. and Mornon, J.P.: Steroid hormone receptor and pharmacology. J. Steroid Biochem. 12:143-157 (1980).

Rayson, B.M. and Lowther, S.O.: Steroid regulation of Na-K-ATPase: Differential sensitivities along the nephron. Am. J. Physiol. 246:F565-F662 (1984).

Rees, A.M. and Bell, P.A.: The involvement of receptor sulfhydryl groups in the binding of steroids to the cytoplasmic glucocorticoid receptor from rat thymus. Biochem. Biophys. Acta 411:121-132 (1975).

Rees, H.D. and Gray H.E.: Glucocorticoids and mineralocorticoids: Actions on brain and behavior. In: Peptides, Hormones and Behavior. (C.B. Nemeroff and A.J. Dunn, Eds.) pp. 533-643 Spectrum Press, New York, NY (1983).

Reich, I.M. and Scott, W.N.: Aldosterone and sodium balance: Towards an understanding of the basic mechanisms. Mount Sinai J. Med. 46:367-377 (1979).

Reich, I.M.; Skipski, I.A. and Scott, W.N.: Mechanisms of hormonal action of ion transport in the toad's urinary bladder. Subcellular localization of aldosterone-induced proteins. Biochim. Biophys. Acta 676:379-385 (1981).

Reichman, M.E.; Foster, C.M.; Eisen, L.P.; Eisen, H.J.; Torain, B.F. and Simons, S.S., Jr.: Limited proteolysis of covalently labeled glucocorticoid receptors as a probe of receptor structure. Biochemistry 23:5376-5384 (1984).

Reker, C.E.; Kovacic-Milivojevic, B.; Eastman-Reks, S.B. and Vedeckis, W.V.: Transformed mouse glucocorticoid receptor: Generation and interconversion of the 3.8 S, monomeric and 5.2 S, oligomeric species. Biochemistry 24:196-204 (1985).

Rettori, O.; Bugyi, H. and Maloney, Jr.J.V.: Effect and mechanism of action of aldosterone treatment on rabbit erythrocytes. Am. J. Physiol. 217:1585-1588 (1969).

Reul, J.M.H.M. and De Kloet, E.R.: Anatomical resolution of two types of corticosterone receptor sites in rat brain with in vitro autoradiography and computerized image analysis. J. Steroid Biochem. 24:269-272 (1986).

Reul, J.M.H.M. and De Kloet, E.R.: Two receptor systems for corticosterone in rat brain: Microdistribution and differential occupation. Endocrinology 117:269-272 (1985).

Richards, J.M. and Swisloki, N.I.: Activation of adenylate cyclase by molybdate. J. Biol. Chem. 254:6857-6860 (1979).

Richardson, K.S.C.; Nowaczynski, W. and Genest, W.: Specific aldosterone-binding proteins in human plasma: Partial characterization. J. Steroid Biochem. 8:951-957 (1977).

Richter, C.P.: Increased salt appetite in adrenalectomized rats. Am. J. Physiol. 115:155-161 (1936).

Richter, C.P.: Salt appetite of mammals: Its dependence on instinct and metabolism. In: L'Instinct dans le Comportment des Animaux et de l'Homme. (M. Autuori, Ed.) Paris, Masson et Cie. pp. 577-632 (1956).

Rossier, B.C.; Paccolat, M.P.; Verrey, F.; Kraehenbuhl, J.-P. and Geering, K.: Mechanism of action of aldosterone: A pleiotropic response. In: Hormones and Cell Regulation vol. 9, pp. 209-225 Elsevier, New York, NY (1985).

Rossier, B.C.; Wilce, P.A. and Edelman, I.S.: Kinetics of RNA labeling in toad bladder epithelium: Effects of aldosterone and related steroids. Proc. Natl. Acad. Sci. U.S.A. 71:3101-3105 (1974).

Rossini, G.P. and Barbiroli, B.: RNAase-sensitive glucocorticoid receptor complexes from HeLa cell nuclei. Biochem. Biophys. Res. Commun. 113:876-882 (1983).

Roth, J. and Taylor, S.: Receptors for peptide hormones: Alteration in diseases of human. Ann. Rev. Physiol. 44:639-651 (1982).

Rousseau, G.G.; Baxter, J.D.; Funder, J.W.; Edelman, I.S. and Tomkins, G.M.: Glucocorticoid and mineralocorticoid receptors for aldosterone. J. Steroid Biochem. 3:219-227 (1972).

Sagnella, G.A.; Markandu, N.D.; Shore, A.C.; Forsling, M.L. and MacGregor, G.A.: Plasma atrial natriuretic peptide: Its relationship to changes in sodium intake, plasma renin activity and aldosterone in man. Clin. Sci. 72:25-30 (1987).

Sakai, R.R.; Nicolaidis, S. and Epstein, A.N.: Salt appetite is supressed by interference with angiotensin II and aldosterone. Am. J. Physiol. 251:R762-R768 (1986).

Sakaue, Y. and Thompson, E.B.: Characterization of two forms of glucocorticoid hormone-receptor complex separated by DEAE-cellulose column chromatography. Biochem. Biophys. Res. Commun. 77:533-541 (1977).

Sandberg, A.A.; Slaunwhite, W.R. and Carter, A.C.: Transcortin: A corticosteroid-binding protein of plasma. III. The effect of various steroids. J. Clin. Invest. 39:1914-1926 (1960).

Sando, J.J.; Hammond, N.D.; Statford, C.A. and Pratt, W.B.: Activation of thymocyte glucocorticoid receptor. J. Biol. Chem. 254:4779-4789 (1979a).

Sando, J.J.; La Forest, A.C. and Pratt, W.B.: ATP-dependent activation of L cell glucocorticoid receptors to the steroid binding form. J. Biol. Chem. 254:4772-4778 (1979b).

Sapirstein, V.S. and Scott, W.N.: Binding of aldosterone by mineralocorticoid-rich cells of the toad urinary bladder. Nature Lond. 257:241-243 (1975).

Sapolsky, R.M.: A mechanism for glucocorticoid toxicity in the hippocampus: Increased neuronal vulnerability to metabolic insults. J. Neurosci. 5:1228-1232 (1985).

Sapolsky, R.M.; Krey, L.C. and McEwen, B.S.: Corticosterone receptors decline in a site-specific manner in the aged rat brain. Brain Res. 289:235-240 (1983).

Sapolsky, R.M.; Krey, L.C. and McEwen, B.S.: Stress down-regulates corticosterone receptors in a site-specific manner in the brain. Endocrinology 114:287-292 (1984).

Sapolsky, R.M.; Krey, L.C. and McEwen, B.S.: Prolonged glucocorticoid exposure reduces hippocampal neuron number: Implications for aging. J. Neurosci. 5:1222-1227 (1985a).

Sapolsky, R.M.; Krey, L.C. and McEwen, B.S.: The neuroendocrinology of stress and aging: The glucocorticoid cascade hypothesis. Endocrine Rev. 7:284-301 (1986).

Sapolsky, R.M.; Meaney, M.J. and McEwen, B.S.: The development of the glucocorticoid receptor system in the rat limbic brain. III. Negative-feedback regulation. Develop. Brain Res. 18:169-173 (1985b).

Sarrieau, A.; Vial, M.; McEwen, B.S.; Broer, Y.; Dussaillant, M.; Pilibert, D.; Moguilewsky, M. and Rostene, W.: Corticosteroid receptors in rat hippocampal sections: Effects of adrenalectomy and corticosterone replacement. J. Steroid Biochem. 24:721-724 (1986).

Scatchard, G.: The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51:600-672 (1949).

Scheidereit, C.; Krauler, P.; von der Ahe, D.; Janich, S.; Rabenau, O.; Cato, A.C.B.; Suske, G.; Westphal, H.M. and Beato, M.: Mechanism of gene regulation by steroid hormones. J. Steroid Biochem. 24:19-24 (1986).

Scholer, D.W.; Mishina, T. and Edelman, I.S.: Distribution of aldosterone receptors in rat kidney cortical tubules enriched in proximal and distal segments. Am. J. Physiol. 237:F360-F366 (1979).

Schulman, G.; Miller-Diener, A.; Litwack, G. and Bastl, C.P.: Characterization of the rat colonic aldosterone receptor and its activation process. J. Biol. Chem. 261:12102-12106 (1986).

Schwartz, G.J. and Burg, M.B.: Mineralocorticoid effects on cation transport by cortical collecting tubules in vitro. Am. J. Physiol. 235:F576-F585 (1978).

Scott, W.N. and Sapirstein, V.S.: Identification of aldosterone-induced proteins in the toad's urinary bladder. Proc. Natl. Acad. Sci. U.S.A. 72:4056-4060 (1975).

Sharp, G.W.G.; Coggins, C.H.; Lichtenstein, N.S. and Leaf, A.: Evidence for a mucosal effect of aldosterone on sodium transport in the toad bladder. J. Clin. Invest. 45:1640-1647 (1966a).

Sharp, G.W.G.; Komack, G.I. and Leaf, A.: Studies on the binding of aldosterone in the toad bladder. J. Clin. Invest. 45:450-459 (1966b).

Sheppard, K.E. and Funder, J.W.: Cortisol 17α acid, transcortin, and the heterogeneity of rat brain glucocorticoid receptors. J. Steroid Biochem. 25:285-288 (1986).

Sherman, M.R.; Moran, M.C.; Tauzon, F.B. and Stevens, Y.-W.: Structure, dissociation, and proteolysis of mammalian steroid receptors. Multiplicity of glucocorticoid receptor forms and proteolytic enzymes in rat liver and kidney cytosols. J. Biol. Chem. 258:10366-10377 (1983).

Shukuya, R. and Schwert, G.: Glutamic acid decarboxylase. III. The activation of the enzyme at low temperatures. J. Biol. Chem. 235:1658-1661 (1960).

Shyamala, G. and Leonard, L.: Inhibition of uterine estrogen receptor transformation by sodium molybdate. J. Biol. Chem. 255:6028-6031 (1980).

Siiteri, P.K.; Murai, J.T.; Hammond, G.L.; Nisker, J.A.; Raymoure, W.J. and Kuhn, R.W.: The serum transport of steroid hormones. Rec. Prog. Hormone Res. 38:457-510 (1982).

Simmen, R.C.M.; Means, A.R. and Clark, J.H.: Estrogen modulation of nuclear matrix-associated steroid-hormone binding. Endocrinology 115:1197-1202 (1984).

Simpson, S.A. and Tait, J.F.: A quantitative method for the bioassay of the effects of adrenal cortical steroids on mineral metabolism. Endocrinology 50:150-161 (1952).

Simpson, S.A. and Tait, J.F.: Recent progress in methods of isolation chemistry and physiology of aldosterone. Rec. Prog. Horm. Res. 11:183-219 (1955).

Simpson, S.A.; Tait, J.F.; Wettstein, A.; Neher, R.; Euw, Jv.; Schindler, O. and Reichstein, T.: Konstitution des aldosterons, des neuen mineralocorticoids. Experientia 10:132-133 (1954).

Slater, E.P.; Anderson, T.; Cattini, P.; Isaacs, R.; Birnbaum, M.J. Gardner, D.G.; Ebernhardt, N.L. and Baxter, J.D.: Mechanisms of glucocorticoid hormone action. Adv. Exp. Med. Biol. 196:67-80 (1986).

Spelsberg, T.C.; Littlefield, B.A.; Seelke, R.; Dani, G.M.; Toyoda, H.; Boyd-Leinen, P.; Thrall, C. and Kon, O.L.: Role of specific chromosomal proteins and DNA sequences in the nuclear binding sites for steroid receptors. Rec. Prog. Hormone Res. 39:463-517 (1983).

Spooner, P.M. and Edelman, I.S.: Further studies on the effect of aldosterone on electrical resistance of toad bladder. Biochim. Biophys. Acta 406:304-314 (1975).

Starka, L.; Hampl, R. and Obenberger, J.: Aldosterone binding in bovine ciliary body. Endocrinol. Exper. 11:203-207 (1977).

Starka, L.; Hampl, R.; Obenberger, J. and Doskocil, M.: The role of corticosteroids in the homeostasis of the eye. J. Steroid Biochem. 24:199-205 (1986).

Stephenson, G.; Hammet, M.; Hadaway, G. and Funder, J.W.: Ontogeny of renal mineralocorticoid receptors and urinary electrolyte response in the rat. Am. J. Physiol. 247:F665-F671 (1984a).

Stephenson, G.; Krozowski, Z.S. and Funder, J.W.: Extravascular CBG-like sites in rat kidney and mineralocorticoid receptor specificity. Am. J. Physiol. 246:F227-F233 (1984b).

Stern, N.; Beck, F. and Sowers, J.: Effect of aldosterone on the human erythrocyte sodium-potassium pump in vitro. Clin. Sci. 64:183-186 (1983).

Stokes, J.B.; Ingram, M.J.; Williams, A.D. and Ingram, D.: Heterogeneity of the rabbit collecting tubule: Localization of mineralocorticoid action to the cortical portion. Kidney Intl. 20:340-347 (1981).

Stricker, E.M.: Thirst and sodium appetite after colloid treatment in rats: Role of the renin-angiotensin-aldosterone system. Behav. Neurosci. 97:725-737 (1983).

Stricker, E.M.: Thirst and sodium appetite after colloid treatment in rats with septal lesions. Behav. Neurosci. 98:356-360 (1984).

Strum, J.M.; Feldman, D.; Taggart, B.; Marver, D. and Edelman, I.S.: Autoradiographic localization of corticosterone receptors Type III in the collecting tubule of the rat kidney. Endocrinology 97:505-516 (1975).

Suyemitsu, T. and Terayama, H.: Specific binding sites for natural glucocorticoids in plasma membranes of rat liver. Endocrinology 96:1499-1508 (1975).

Suzuki, S.: Carbonic anhydrase, Mg^{2+}-HCO_3^--ATPase and Mg^{2+}-Na^+-K^+-ATPase in rat intestinal mucosa: Effects of adrenalectomy and aldosterone administration. J. Steroid Biochem. 14:449-456 (1981).

Suzuki, S. and Ozaki, N.: Activating effect of aldosterone on carbonic anhydrase activity of rat small intestinal mucosa. Ann. N.Y. Acad. Sci. 429:306-308 (1984).

Suzuki, S.; Ozaki, N.; Yashida, J.; Takamura, S.; Takeuchi, Y. and Kudo, S.: Brush border Mg^+-HCO_3^--ATPase, supernatant carbonic anhydrase and other enzyme activities isolated from rat intestinal mucosa: effect of adrenalectomy and aldosterone administration. J. Steroid Biochem. 19:1419-1433 (1983).

Svec, F.: Glucocorticoid receptor regulation. Life Sci. 36:2359-2366 (1985a).

Svec, F.: Biopotency of corticosterone and dexamethasone in causing glucocorticoid downregulation. J. Steroid Biochem. 23:69-671 (1985b).

Svec, F. and Rudis, M.: Glucocorticoids regulate the glucocorticoid receptor in the AtT-20 cell. J. Biol. Chem. 256:5984-5987 (1981).

Swaneck, G.E.; Chu, L.L.H. and Edelman, I.S.: Stereospecific binding of aldosterone to renal chromatin. J. Biol. Chem. 245:5382-5389 (1970).

Takagi, M.; Franco-Saenz, R. and Mulrow, P.J.: Effect of atrial natriuretic factor on the plasma aldosterone response to potassium infusion in rats - in vivo study. Life Sci. 39:359-364 (1986).

Tashima, Y.; Terui, M.; Itoh, H.; Shima, H.; Mizunuma, H. and Kobayashi, R.: Sulfhydryl groups of aldosterone receptors from swine kidney. Biochem. Biophys. Res Commun. 124:51-56 (1984).

Thorn, G.W.; Dorrance, S.S. and Day, E.: Addison's disease: Evaluation of synthetic deoxycorticosterone acetate therapy in 158 patients. Ann. Intl. Med. 16:1053-1096 (1942).

Timasheff, S.N.; Lee, J.C.; Pitz, E.P. and Tweedy, N.: The interaction of tubulin and other proteins with structure stabilizing solvents. J. Colloid Interface Sci. 55:658-663 (1976).

Toft, D.O. and Nishigori, H.: Stabilization of the avian progesterone receptor by inhibitors. J. Steroid Biochem. 11:413-416 (1979).

Tornello, S.; Orti, E.; De Nicola, A.F.; Rainbow, T.C. and McEwen, B.S.: Regulation of glucocorticoid receptors in brain by corticosterone treatment of adrenalectomized rats. Neuroendocrinology 35:411-417 (1982).

Towle, A.C. and Sze, P.Y.: Steroid binding to synaptic plasma membrane: Differential binding of glucocorticoids and gonadal steroids. J. Steroid Biochem. 18:135-143 (1983).

Trachewsky, D; Oakes, M.L.I. and Morris, D.J.: Induction of flavokinase (EC 2.7.1.26) by aldosterone in the rat kidney. Endocrinology 116:879-888 (1985).

Turner, B.B.: Tissue differences in the up-regulation of glucocorticoid-binding proteins in the rat. Endocrinology 118:1211-1216 (1986).

Tymoczco, J.L. and Lee, J.H.: Chymotrypsin treatment of glucocorticoid receptor attenuates RNA-dependent inhibition of DNA binding. Evidence for a distinct RNA-binding site. Biochim. Biophys. Acta 846:193-199 (1985).

Tymoczco, J.L. and Phillips, M.M.: The effects of ribonuclease on rat liver dexamethasone receptor: Increased affinity for deoxyribonucleic acid and altered sedimentation profile. Endocrinology 42:142-149 (1983).

Tymoczco, J.L.; Phillips, M.M. and Vernon, S.M.: Binding of the rat liver 7-8 S dexamethasone receptor to deoxyribonucleic acid. Arch. Biochem. Biophys. 230:345-354 (1984).

Valeri, P.; Angelucci, L. and Palmer, M.: Specific [^{3}H]corticosterone uptake in the hippocampus and septum varies with social settings in mice: Hormone-receptor autoregulation may be involved. Neurosci Lett. 9:249-254 (1978).

Vandewalle, A.; Farman, N.; Bencsath, P. and Bonvalet, J.P.: Aldosterone binding along the rabbit nephron: an autoradiographic study on isolated tubules. Am. J. Physiol. 240:F172-F179 (1981).

Veldhuis, H.D.; De Kloet, E.R.; von Zoest, I. and Bohus, B.: Adrenalectomy reduces exploratory activity in the rat: A specific role of corticosterone. Horm. Behav. 16:191-198 (1982a).

Veldhuis, H.D.; Koppen, C.V.; Ittersum, M.V. and De Kloet, E.R.: Specificity of the adrenal steroid system in rat hippocampus. Endocrinology 110:2044-2051 (1982b).

Vesely, D.L.: On the mechanism of action of adrenocortical steroids: Cortisol and aldosterone enhance guanylate cyclase activity. J. Pharmacol. Exp. Ther. 214:561-566 (1980).

Voute, C.L. and Meier, W.: The mitochondria-rich cell of frog skin as hormone sensitive "shunt-path." J. Memb. Biol. 40:151-165 (1978).

Walters, M.R.: Steroid hormone receptors and the nucleus. Endocrine Rev. 6:512-543 (1985).

Walters, M.R. and Clark, J.H.: Relationship between the quantity of progesterone receptor and the antagonism of estrogen-induced uterotropic response. Endocrinology 105:382-386 (1979).

Webb, M.L.; Schmidt, T.J., Robertson, N.M. and Litwack, G.: Evidence for an association of a ribonucleic acid with purified, unactivated glucocorticoid receptor. Biochem. Biophys. Res. Commun. 140:204-211 (1986).

Weiser, J.N.; Do, Y.S. and Feldman, D.: Synthesis and secretion of corticosteroid-binding globulin by rat liver. J. Clin. Invest. 68:461-467 (1979).

Weisinger, R.S.; Considine, P.; Denton, D.A. and McKinley, M.J.: Rapid effect of change in cerebrospinal fluid sodium concentration on salt appetite. Nature Lond. 280:490-491 (1979).

Weisinger, R.S. and Woods, S.C.: Aldosterone-elicited sodium appetite. Endocrinology 89:538-544 (1971).

Weiss, J.M. and Gray, P.: Hormones and avoidance behavior: A different approach points to a role for mineralocorticoids. Prog. Brain Res. 39:471-480 (1973).

Weisz, A.; Baxter, J.D. and Lan, N.C.: Interaction of sodium molybdate with highly purified glucocorticoid receptor. J. Steroid Biochem. 20:289-293 (1984).

Welshons, W.V.; Krummel, B.M. and Gorski, J.: Nuclear localization of unoccupied receptors for glucocorticoids, estrogens, and progesterone in GH_3 cells. Endocrinology 117:2140-2147 (1985).

Welshons, W.V.; Lieberman, M.E. and Gorski, J.: Nuclear localization of unoccupied oestrogen receptors. Nature Lond. 307:747-749 (1984).

Werthamer, S.; Samuels, A.J. and Amaral, L.: Identification and partial purification of "transcortin"-like protein within human lymphocytes. J. Biol. Chem. 248:6398-6407 (1973).

Westphal, U.: Corticosteroid-binding globulin. A review of some recent aspects. Mol. Cell. Biochem. 55:145-157 (1983).

Wheeler, R.H.; Leach, K.L.; Laforest, A.C.; O'toole, T.E.; Wagner, R. and Pratt, W.B.: Glucocorticoid receptor activation and inactivation in cultured human lymphocytes. J. Biol. Chem. 256:434-441 (1981).

Will, P.C.; Cortright, R.N.; Delisle, R.C.; Douglas, J.G. and Hopfer, U.: Regulation of amiloride-sensitive electrogenic sodium transport in the rat colon by steroid hormones. Am. J. Physiol. 248:G124-G132 (1985).

Williamson, H.E.: Mechanism of antinatriuretic action of aldosterone. Biochem. Pharm. 12:1449-1450 (1963).

Wilson, E.M.; Wright, B.T. and Yarbrough, W.G.: The possible role of disulfide bond reduction in transformation of the 10S androgen receptor. J. Biol. Chem. 261:6501-6508 (1986).

Wilson, K.M.; Sumners, C.; Hathaway, S. and Fregly, M.J.: Mineralocorticoids modulate central angiotensin II receptors in rats. Brain Res. 382:87-96 (1986).

Wolf, G. and Di Cara, L.V.: Impairments in sodium appetite after lesions of gustatory thalamus: Replication and extension. Behav. Biol. 10:105-112 (1974).

Wolf, G.; Di Cara, L.V. and Braun, J.J.: Sodium appetite in rats after neocortical ablation. Physiol. Behav. 5:1265-1269 (1970).

Wolf, G. and Handal, P.J.: Aldosterone-induced sodium appetite: Dose response and specificity. Endocrinology 78:1120-1124 (1966).

Woodcock, E.A.; McLeod, J.K. and Johnston, C.I.: Vasopressin stimulates phosphatidylinositol turnover and aldosterone synthesis in rat adrenal glomerulosa cells: comparison with angiotensin II. Endocrinology 118:2432-2436 (1986).

Woodburry, D.M.: Relation between the adrenal cortex and the central nervous system. Pharmacol. Rev. 10:275-357 (1958).

Wrange, O. and Yu, Z.-Y.: Mineralocorticoid receptor in rat kidney and hippocampus: Characterization and quantitation by isoelectric focusing. Endocrinology 113:243-250 (1983).

Wrange, O.; Carlstedt-Duke, J. and Gustafsson, J.-A.: Stoichiometric analysis of the specific interaction of the glucocorticoid receptor with DNA. J. Biol. Chem. 261:11770-11778 (1986).

Wright, F.S. and Giebisch, G.H.: Renal potassium transport: Contribution of individual nephron segments and populations. Am. J. Physiol. 235:F515-F527 (1978).

Yang, C.-P.H.; Skipski, I.A. and Scott, W.N.: Cell-free translation of RNA isolated from aldosterone-treated toad bladder mucosal cells. Biochim. Biophys. Acta 654:86-93 (1981).

Yang, C.-R.; Mester, J.; Wolfson, A.; Renoir, J.-M. and Baulieu, E.-E.: Activation of the chick oviduct progesterone receptor by heparin in the presence or absence of hormone. Biochem. J. 208:399-406 (1982).

Yongue, B.G. and Roy, E.J.: Radioimmunoassay of hippocampal cell-nuclear aldosterone and corticosterone. Soc. Neurosci. Abstr. 10:478 (1984).

Young, D.B.: Long term effects of aldosterone on potassium distribution. Fed. Proc. 38:969 (1979).

Young, D.B. and Jackson, T.E.: Effect of aldosterone on potassium distribution. Am. J. Physiol. 243:R526-R530 (1982).

Zager, P.G.; Burtis, W.J.; Luetscher, J.A.; Dowdy, A.J. and Sood, S.: Increased plasma protein binding and lower metabolic clearance rate of aldosterone in plasma of low cortisol concentration. J. Clin. Endocrinol. Met. 42:207-214 (1976).

Zamir, N.; Gutman, Y. and Ben-Ishay, D.: Experimental hypertension and catecholamine distribution in the rat brain. Brain Res. 171:101-112 (1979).

Zipser, R.D.; Meidar, V. and Horton, R.: Characteristics of aldosterone binding in human plasma. J. Clin. Endocrinol. Met. 50:158-162 (1980).

Ziegels, J.: The vertebrate subcommissural organ: A structural and functional review. Arch. Biol. 87:429-476 (1976).

BIOGRAPHICAL SKETCH

The author was born on April 22, 1958, in Sari, Iran--a city of about 100,000 population located 18 miles south of the Caspian Sea. The author received his elementary and high school education as well as several years of private formal training in painting and graphic arts in Sari. Following graduation from Payam High School (May 1976), after careful consideration, his parents decided to send him to the United States for higher education training. Because of the author's primary interest in Medicine, he entered Phillips University in Enid, Oklahoma, as a Pre-Med student in the Fall of 1977.

In addition to academic achievements (e.g.; Outstanding Biology Student award, 1979; research grant in the amount of $6,000, 1980; etc.) at Phillips University, the author advanced to the presidency of the local chapter of The Blue Key National Honor Fraternity (1981-82). As part of his formal educational training, the author attended Harvard University during summer of 1980. He graduated cum laude from Phillips University with a Bachelor of Arts in biology and chemistry in May of 1982. While a student at Phillips University, the author worked as a urology and orthopedics technician at a local hospital.

After failing to gain admission for an M.D.-Ph.D. program, with the advice of his undergraduate mentor, the author decided to obtain his Ph.D. degree first and then apply to a medical school. Although he was admitted to several graduate schools, due to his genuine interest in neuroendocrinology, the author chose to accept the offer from the Department of Neuroscience, University of Florida, where he has been a

Ph.D. candidate since August 1982. The author's honors and accomplishments during the past 5 years include research assistantship (1982-85), research fellowship (1985-87), presentation at a number of local and national meetings and several full-length articles of his work in a number of scientific refereed journals.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William G. Luttge, Chairman
Professor of Neuroscience

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Adrian J. Dunn
Professor of Neuroscience

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Melvin J. Fregly
Graduate Research
Professor of Physiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Marieta B. Heaton
Associate Professor of
Neuroscience

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Kathleen T. Shiverick
Associate Professor of
Pharmacology and
Therapeutics

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philisophy.

May 1987

Dean, College of Medicine

Dean, Graduate School

UNIVERSITY OF FLORIDA
3 1262 08554 4012